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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica do Professor Doutor Jorge Filipe de Almeida Vieira Lima, Investigador do IPATIMUP e co-orientação da Doutora Sandra Isabel Moreira Pinto Vieira, Professora da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro.

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palavras-chave

Feocromocitoma, paraganglioma, *SDH*, *RET, VHL, NF1, TMEM127*, *MAX*, mutações, imunohistoquímica

resumo

Feocromocitomas (FEO) e paragangliomas (PGL) são tumores neuroendócrinos raros que derivam do tecido paragangliónico do sistema nervoso autónomo, surgindo mais especificamente da medula adrenal – feocromocitomas – ou da paraganglia extra-adrenal – paragangliomas. Estes tumores são normalmente esporádicos, mas podem ocorrer como parte de diferentes síndromes tumorais hereditárias.

Até à data, foram identificados dez genes que predispõem ao desenvolvimento de FEO e PGL: genes do complexo succinato desidrogenase (*SDHA*, *SDHB*, *SDHC* e *SDHD*); cofactor do complexo SDH (*SDHAF2*); *RET*, *VHL*, *NF1*, *TMEM127* e *MAX*.

O objectivo deste trabalho consistiu na identificação de alterações genéticas causais em duas séries de pacientes com FEO e PGL. Para tal, foi feita a pesquisa de alterações nos genes *SDH*, *SDHAF2*, *RET*, *VHL*, *TMEM127* e *MAX*. Para além disso, foi analisada a expressão da proteína SDH por imunohistoquímica.

Numa série composta apenas por FEO's, a maioria dos pacientes foram identificados como esporádicos (82%), enquanto dois apresentavam síndrome MEN2 (4%) com mutações no gene *RET*, quatro com NF1 (7%) e um com VHL (2%); para além destes, três casos (5%) mostraram ausência de expressão de SDH, um deles com mutação. Não foram encontradas alterações genéticas patogénicas nos genes *TMEM127* e *MAX*.

Numa série de casos Portugueses constituída por FEO's e PGL's, seis pacientes (50%) foram identificados com mutação: quatro no gene *SDHB* e um no gene *SDHD*; adicionalmente, uma mutação patogénica foi identificada no gene *MAX*. Toda a informação clínica, genética e imunohistoquímica foi relacionada, provando que um diagnóstico baseado em estudos genéticos e imunohistoquímicos constitui o melhor método para pacientes com FEO/PGL's.

keywords

Pheochromocytoma, paraganglioma, *SDH*, *RET, VHL, NF1, TMEM127*, *MAX*, mutations, immuhistochemistry

abstract

Pheochromocytomas (PCC) and paragangliomas (PGL) are rare neuroendocrine tumours derived from the paraganglionic tissue of the autonomic nervous system, arising more specifically from adrenal medulla - pheochromocytomas - or from extra-adrenal paraganglia - paragangliomas. These tumours are usually sporadic, but can occur as part of different hereditary tumour syndromes.

To date, ten genes have been identified to predispose to the development of PCC and PGL: succinate dehydrogenase (SDH) complex subunit genes (*SDHA*, *SDHB*, *SDHC* and *SDHD);* SDH complex cofactor *SDHAF2; RET*, *VHL, NF1, TMEM127* and *MAX*.

The aim of this work was to identify the causative genetic defects in two series of PCC and PGL patients. For this purpose, genetic screening was performed for *SDH, SDHAF2, RET, VHL,TMEM127* and *MAX* genes. Also, SDH protein expression was assessed by immunohistochemistry.

In a series composed only of PCC's, the majority of patients were sporadic (82%), while two had MEN2 (4%) with *RET* mutations, four had NF1 (7%) and one patient presented VHL (2%); furthermore, three patients (5%) showed absence of SDH, one with mutation. Pathogenic genetic defects in *TMEM127* and *MAX* were not found.

In a Portuguese series comprising both PCC and PGL's, six patients (50%) were found to have germline mutations: four in *SDHB* and one in *SDHD;* additionally, one pathogenic mutation was identified in *MAX* gene. Clinical, genetic and immunohistochemical data was associated, proving that a diagnosis based on genetic and immunohistochemical studies is a suitable approach for the management of PCC/PGL's patients.

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1. GLOSSARY

ANS - Autonomic Nervous System;

ATP - Adenosine triphosphate;

bHLHZip - Basic helix-loop-helix leucine zipper;

COX - Cytochrome c oxidase;

CS - Citrate synthase;

CybL - Cytochrome b succinate dehydrogenase large subunit;

CybS - Cytochrome b succinate dehydrogenase small subunit;

DNA - Desoxyribonucleic acid;

dNTP - Deoxyribonucleotide triphosphate;

FAD – Flavin adenine dinucletotide;

FADH2 – Flavin adenine dinucleotide (reduced form);

GDNF - Glial cell line-derived neurotropic factor family;

GIST - Gastrointestinal stromal tumours;

GTP - Guanosine triphosphate;

HIF - Hypoxia inducible factor;

LOH – Loss of heterozygosity;

MAX - Myc-associated factor X;

MEN2 – Multiple endocrine neoplasia type 2;

MLPA - Multiplex ligation-dependent probe amplification;

MTC - Medullary thyroid carcinoma;

mtDNA – Mitochondrial DNA;

mTOR - Mechanistic target of rapamycin;

NAD – Nicotinamide adenine dinucleotide;

NADH – Nicotinamide adenine dinucleotide (reduced form);

NF1 – Neurofibromatosis type 1;

PCC – Pheochromocytoma;

PCR – Polymerase chain reaction;

PGL – Paraganglioma;

PHD - Prolyl hydroxylases;

PSNS - Parasympathetic nervous system;

PTC - Papillary thyroid carcinoma;

RCC - Renal cell carcinoma;

RET – Rearranged during transfection;

Rheb - Ras homolog enriched in brain;

ROS - Reactive oxygen species;

SDH - Succinate dehydrogenase;

SDHA – Succinate dehydrogenase subunit A;

SDHAF1 - Succinate dehydrogenase complex assembly factor 1;

SDHAF2 - Succinate dehydrogenase complex assembly factor 2;

SDHB – Succinate dehydrogenase subunit B;

SDHC – Succinate dehydrogenase subunit C;

SDHD – Succinate dehydrogenase subunit D;

SNP – Single nucleotide polymorphism;

SNS - Sympathetic nervous system;

S6K - Ribosomal protein S6 kinase;

TCA – Tricarboxylic acid cycle;

TMEM127 - Transmembrane protein 127;

tRNA – Transfer RNA;

TSC - Tuberous sclerosis protein;

UPD – Uniparental disomy;

VDAC - Voltage-dependent anion channel;

VEGF - Vascular endothelial growth factor;

VHL – Von Hippel-Lindau;

4EBP1 - Eukaryotic translation initiation factor 4E binding protein 1.

2. INTRODUCTION

PHEOCHROMOCYTOMAS, PARAGANGLIOMAS AND THE AUTONOMIC NERVOUS SYSTEM

Pheochromocytomas (PCC) and paragangliomas (PGL) are rare neuroendocrine tumours derived from the paraganglionic tissue of the Autonomic Nervous System (ANS) (DeLellis 2004).

The ANS comprises the sympathetic (SNS) and parasympathetic (PSNS) divisions forming the neural circuitry and working with neuroendocrine system for homeostasis regulation and body's physiology control. The SNS enhances catabolic activities specially energy mobilization, whereas PSNS stimulates anabolic processes like promoting rest and reducing energy use (Squire et al. 2008).

The SNS and PSNS are organized in pre-ganglionic neurons with cell bodies within the central nervous system (brain stem and spinal cord) and post-ganglionic motor neurons that project to the effector tissues. The SNS has pre-ganglionic neurons in the thoracic and lumbar regions of the spinal cord (T1 – L2) that in turn project to postganglionic motor neurons in paravertebral, prevertebral autonomic ganglia and chromaffin cells of adrenal medulla; the PSNS has preganglionic neurons in brain stem (cranial nerves III, VII, IX, X) and in the sacral spinal cord (S2, S3, S4) that project to postganglionic motor neurons in ganglia near or inside the viscera (Squire et al. 2008).

The adrenal medulla is the one exception to the two-neuron pathway rule since post-ganglionic nerve cells are classical neurosecretory cells. They secrete postganglionic sympathetic transmitters, catecholamines, directly into the bloodstream (Nussey et al. 2001; Squire et al. 2008).

The adrenal glands are two small organs with 4-5g of weight, located on the top of the kidneys with a pyramidal form, extending toward the hilum of each kidney. The arterial blood supply is done by branches from the aorta, the inferior phrenic artery and the renal artery (Kay et al. 2008).

These organs are organized in two different structural and functional parts: the adrenal cortex and the adrenal medulla. The adrenal cortex secretes mainly mineralocorticoids (aldosterone), glucocorticoids (cortisol) and androgen. The adrenal medulla, through direct stimulation by acetylcholine from sympathetic nerves, secretes catecholamines - epinephrine (80%), norepinephrine (20%) and minimal amounts of dopamine (Kay et al. 2008). Catecholamines modulate stress response, metabolism and blood pressure (DeLellis 2004).

The adrenal medulla constitutes less than 20% of the adrenal gland and it's composed by chromaffin cells. These are polygonal cells, organized in cords and with neural crest origin, which synthesize and secrete epinephrine and norepinephrine in response to stimulation of pre-ganglionic cholinergic sympathetic nerves. Thus, they are considered modified post-ganglionic sympathetic neurons and contain secretory granules for catecholamines. According to the hormone synthetized these cells can be divided in two types: cells containing epinephrine (the majority of chromaffin cells) that stimulates glycogenolysis, lipolysis and inhibits insulin secretion; cells containing norepinephrine (minority of the chromaffin cells) that stimulates α- and β-adrenergic receptors in cells causing higher blood pressure, heart rate and cardiac contractility (Dudek 2000; Kuehnel 2003; Kay et al. 2008). They receive blood from the venules of the cortex rich in cortisol that in turn drain to medullary venules, or directly from medullary arterioles. The name "chromaffin" comes from the tumour tissue affinity for chromate salts, leading to characteristic staining (Nussey et al. 2001; Linos et al. 2005 ; Welander et al. 2011).

The paraganglionic system has an origin in the neural crest tissue, during early gestation, becoming very similar in terms of histological features. This tissue comprises two parts: the adrenal medulla and the diffuse extra-adrenal paraganglia. Additionally, the extra-adrenal paraganglia is organized in sympathoadrenal and parasympathetic paraganglia: the sympathetic paraganglia comprises the axial regions of the trunk along the prevertebral and paravertebral sympathetic chains from the neck near the superior cervical ganglion to the abdomen and pelvis, and also the connective tissue within or near pelvic organs like bladder and prostate gland; the parasympathetic paraganglia is almost entirely restricted to the head and neck parasympathetic nerves and upper mediastinum, being the most common location the carotid body. The carotid body functions as detector of alterations in oxygen and carbon dioxide partial pressure in the arterial blood, as well as sensor of pH and temperature. Other locations of parasympathetic paraganglia include the jugulotympanic ganglion, along the glossopharyngeal and vagus nerves and more rarely the orbit, nasal cavity, paranasal sinuses, nasopharynx, larynx, trachea and thyroid (Nussey et al. 2001; DeLellis 2004; Linos et al. 2005 ; Welander et al. 2011).

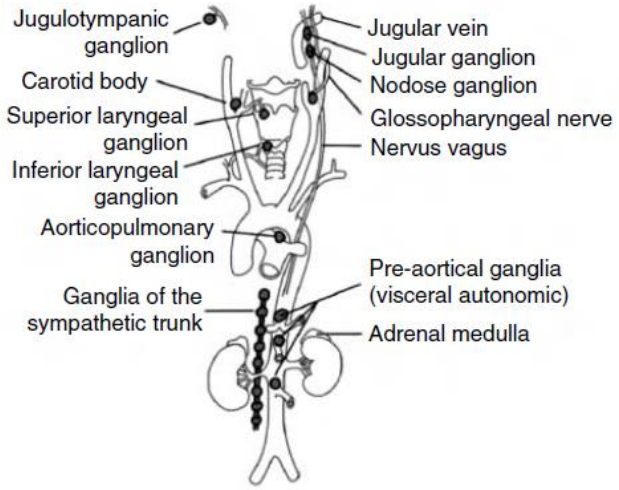


Figure 1 – Anatomical structures of paraganglia (Welander et al. 2011).

Tumours arising from adrenal medulla are termed pheochromocytomas, whereas tumours from extra-adrenal paraganglia are named extra-adrenal paragangliomas, or only paragangliomas, defined by the World Health Organization (Welander et al. 2011).

Although PCC and PGL are indistinguishable at the cellular level, several differences are found concerning the anatomic distribution and secretory products (DeLellis 2004).

EPIDEMIOLOGY OF PCC AND PGL

PCC and PGL are rare tumours with an annual incidence of two to ten cases per million people. Although occurring in all ages, they are most frequent between 30 and 50 years, with a similar distribution between genders (DeLellis 2004). These tumours are often benign, but associated with high morbidity and mortality due to a mass effect and high levels of circulating catecholamines (Welander et al. 2011; Fishbein et al. 2012).

In terms of relative incidence of the different tumour types, PCC is the most prevalent tumour, followed by sympathetic PGL and parasympathetic PGL (Fishbein et al. 2012).

In 2002 a graded score was created, in order to help predicting potential malignancy of PCC/PGL based in histological findings of the tumours (vascular density, amount of necrosis, among other features); nevertheless, it was considered unreliable due to variability (Welander et al. 2011). Nowadays, the WHO guidelines define malignant PCC/PGL as a metastatic lesion to sites where chromaffin tissue is not normally present (Fishbein et al. 2012).

The presence of distant metastases, mainly in bone, liver and lung tissue occurs, approximately, in 5-13% of PCC, 15-23% of sympathetic PGL and 2-20% of parasympathetic PGL (Bardella et al. 2011; Fishbein et al. 2012).

SYMPTOMS

PCC and sympathetic PGL have histological and functional similarities (Welander et al. 2011; Fishbein et al. 2012). A deregulation in synthesis and secretion of catecholamines by chromaffin cells of the paraganglionic tissue leads to large amounts of circulating hormones – epinephrine, norepinephrine, dopamine – at rates many times higher than normal (Welander et al. 2011). PGL of parasympathetic origin, in contrast, is a nonfunctional tumour, generally nonsecretory (DeLellis 2004; Welander et al. 2011).

The excessive catecholamine secretion in PCC's and sympathetic PGL's can cause hypertension, severe cardiovascular and neurological manifestations such as seizures, shock, heart failure, cardiac arrhythmias and stroke and even death. The main symptoms include headache, palpitations, diaphoresis, pallor, anxiety, nausea, tremors, abdominal and chest pain, although some patients (10%) have minor or no symptoms (DeLellis 2004; Welander et al. 2011). These symptoms become worse and more frequent with tumour progression (Blake et al. 2011; Welander et al. 2011; Fishbein et al. 2012).

PGL of parasympathetic origin presents as a slow-growing, painless cellular mass, frequently asymptomatic (Blake et al. 2011). Nevertheless, the compression of vital organs by the tumour mass can lead to the appearance of uncomfortable symptoms (DeLellis 2004; Welander et al. 2011).

GENETICS

PCC and PGL are usually sporadic, but they frequently occur as part of several different hereditary tumour syndromes (Bardella et al. 2011). To date, ten genes have been identified as predisposing to the development of PCC and PGL, making these the tumours with the largest number of associated genes (Fishbein et al. 2012).

Genetic screening in patients with PCC or PGL had not been considered until the year 2000, when only about 10% of these tumours were classified as hereditary cases as part of multiple tumour syndromes. Only recently the genetic screening for hereditary disease was recommended, since the frequency of germline mutations increased to about one third of all PCC and PGL cases (Fishbein et al. 2012).

The most studied genes are the succinate dehydrogenase (SDH) complex subunit genes that comprise *SDHA*, *SDHB*, *SDHC* and *SDHD* genes as well as the SDH complex cofactor *SDHAF2.* Moreover, recent susceptibility genes have been linked with PCC and PGL development - *TMEM127* and *MAX* (Iacobone et al. 2011; Jafri et al. 2012). Three additional PCC and PGL susceptibility genes are associated with well characterized familial syndromes - *RET* gene for multiple endocrine neoplasia type 2 (MEN2), *VHL* gene for von Hippel-Lindau disease (VHL) and *NF1* gene for neurofibromatosis type 1 (NF1) (Fishbein et al. 2012).

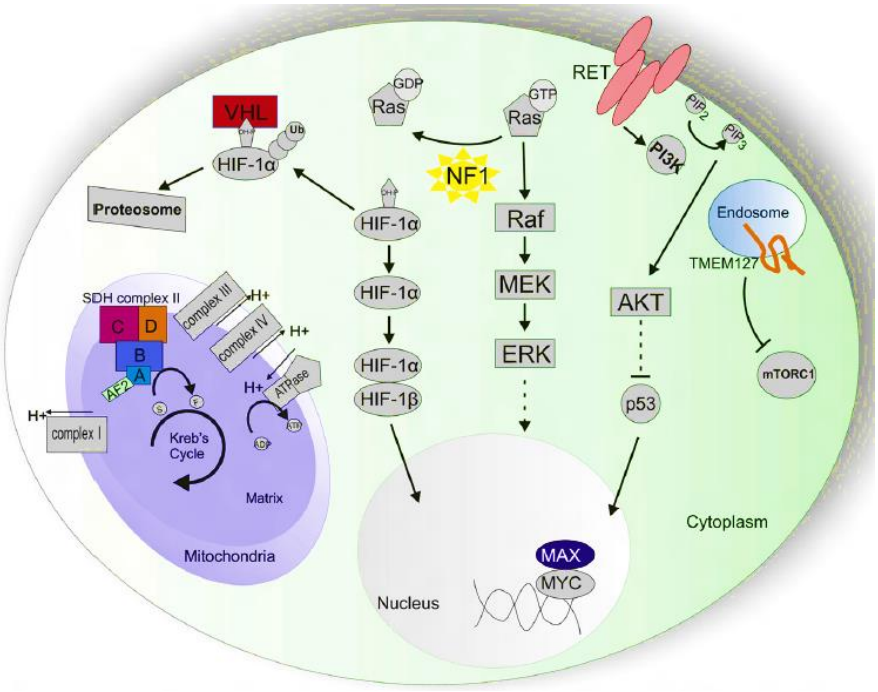


Figure 2 – Signalling pathways for the ten genes associated with PCC/PGL's development (Fishbein et al. 2012).

SDH COMPLEX AND MITOCHONDRIA

Mutations in any of the succinate dehydrogenase (SDH) complex genes that encode complex II, the simplest of the electron transport chain, cause familial PCC/PGL syndromes. The SDH enzyme is a highly conserved heterotetrameric protein that comprises four subunits - SDHA, SDHB, SDHC and SDHD. These four subunits are encoded by homonymous nuclear genes and imported to the mitochondria where they undergo modifications, folding and assembly (Fishbein et al. 2012).

The mitochondrion is an organelle surrounded by a double-membrane - the inner and the outer mitochondrial membranes – separated by an intermembrane space and with an internal matrix. The inner membrane constitutes numerous folds – cristae – which extend to the matrix of the organelle. Each component plays a different function in the organelle with matrix and inner membrane as the most important compartments of mitochondria (Bardella et al. 2011; Welander et al. 2011; Fishbein et al. 2012). Typically, mammalian cells contain hundred mitochondria and liver cells more than a thousand (Cooper 2000; Alberts et al. 2002).

Mitochondria play a critical role in the generation of metabolic energy in eukaryotic cells. The main processes performed in mitochondria are the Krebs cycle, the β-oxidation pathway of fatty acid metabolism and oxidative phosphorylation. The aim of these pathways is the production of ATP, the useful energy for all the metabolic processes performed in the human body, as well as intermediate metabolites that can be used in anabolic metabolism (Metzler 2004).

The breakdown of glucose and fatty acids by oxidative reactions allows the formation of metabolic energy. In the initial stage of glucose metabolism, named glycolysis, glucose is converted in pyruvate in the cytosol. This glycolysis product is then transported into the mitochondrial matrix where it suffers a series of oxidations termed as the Krebs cycle. Pyruvate is oxidized to acetyl CoA and consequently broken down to CO_2 by specific enzymes located in the matrix of mitochondria. Concomitantly, NAD^+ (nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotide) are reduced to NADH and $FADH_2$, respectively, and the high-energy electrons from these products are transferred to a series of carriers in the inner mitochondrial membrane in order to achieve the oxidative phosphorylation. The energy formed along the transfer reactions is then converted in potential energy to generate ATP (Cooper 2000; Metzler 2004).

The electron transport chain has many components, particularly cytochromes a, a_3, b, c and c_1 as well as flavins, ubiquinone and nonheme iron. Furthermore, this pathway consists of five complexes that catalyze different reactions in the electron transport process: NADH-ubiquinone oxidoreductase (complex I), succinateubiquinone oxidoreductase (complex II), ubiquinolcytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V) (Cooper 2000).

All the complexes are linked by ubiquinone and cytochrome c, two soluble electron carriers (Metzler 2004).

As previously mentioned, *SDH* comprises four genes. The gene *SDHA* encodes the flavin protein with 70kDa that binds FAD as cofactor in the catalytic core allowing the binding to succinate (Metzler 2004). *SDHB* encodes the other part of the catalytic domain which also forms an interface with the membrane anchor. The protein encoded is an iron protein of 28 kDa that contains three iron-sulphur clusters (2Fe-2S, 3Fe-4S and 4Fe-4S) for electron transport from flavin to the final acceptor ubiquinone (Horsefield et al. 2006; Welander et al. 2011; Fishbein et al. 2012).

These two proteins, SDHA and SDHB, form a peripheral complex attached to the inner membrane of the mitochondria through two hydrophobic membrane proteins encoded by *SDHC* and *SDHD* genes (Horsefield et al. 2006; Welander et al. 2011; Fishbein et al. 2012). SDHC and SDHD proteins comprise three membrane helices, a histidine sidechain for binding a b-type cytochrome and two ubiquinone-binding sites (Horsefield et al. 2006; Welander et al. 2011; Fishbein et al. 2012).

The SDH mitochondrial enzyme complex is the only one involved both in the electron transport chain and in the Krebs cycle (Smeitink 2004; Horsefield et al. 2006). The main functions of SDH enzyme include catalyzing the oxidation of succinate to fumarate in the Krebs cycle and also transferring electrons to the terminal acceptor ubiquinone or coenzyme Q in the electron transport chain, reducing it to ubiquinol (Horsefield et al. 2006; Fishbein et al. 2012).

Germline mutations in nuclear genes encoding SDH subunits linked for the first time a genetic mitochondrial defect and tumour development, in this case PCC and PGL (Horsefield et al. 2006; Welander et al. 2011; Fishbein et al. 2012).

The initial linkage analysis studies identified three PGL susceptibility loci on chromosomes 11 and 1, which were categorized as “paraganglioma locus 1” (PGL1) on 11q23, “paraganglioma locus 2” on 11q13.1 and “paraganglioma locus 3” on 1q21 (Bardella et al. 2011).

In 2000, gene mapping studies allowed the identification of S*DHD* as the PGL1 gene; patients with familial PGL’s were found to harbour germline loss-of-function *SDHD* mutations, giving rise to the link between *SDH* and neuroendocrine tumours (Bardella et al. 2011). *SDHD* mutations were thereafter discovered in familial and apparently sporadic PCC/PGL’s (Bardella et al. 2011; Welander et al. 2011). Soon after, *SDHB* germline mutations (PGL4) were identified in both familial PCC’s and PGL’s, followed by *SDHC* mutations (PGL3) in PGL (Welander et al. 2011). In contrast, mutations in the *SDHA* gene were only identified in 2010, when a patient with PGL and another with PCC were found to harbour a germline *SDHA* mutation (Bardella et al. 2011; Welander et al. 2011).

In 2009, two genes - *SDHAF1* and *SDHAF2* – which encode succinate dehydrogenase complex assembly factors 1 and 2 were discovered and *SDHAF2* gene mutations (PGL2) were associated with PGL’s but not with PCC’s patients (Bardella et al. 2011; Welander et al. 2011). These two factors are involved in the assembly of the SDH complex (Karasek et al. 2010; Welander et al. 2011).

SDH genes function as tumour suppressors with alterations such as missense, nonsense, frameshift and splice-site mutations (Welander et al. 2011). The genetic variants in *SDH* genes that predispose to PCC/PGL are germline heterozygous mutations with LOH of the non-mutated allele in the tumour tissue that leads to suppression of SDH enzyme activity and an absence of protein expression, resulting in neoplastic transformation (Welander et al. 2011). The most commonly mutated genes are *SDHB* and *SDHD* (Bardella et al. 2011; Welander et al. 2011).

The incidence of PCC/PGL syndrome is estimated to be between 1/50 000 and 1/20 000 (Welander et al. 2011). It is estimated that, by the age of 35, 38% to 60% of individuals with mutations in *SDHD*, *SDHB* and *SDHC* genes will develop PCC/PGL's (Welander et al. 2011). Patients with inherited susceptibility to PCC/PGL's present more frequently aggressive tumours and increased risk of metastasis and mortality (Bardella et al. 2011).

Family history of PCC/PGL, multifocal disease, younger age at onset and malignant tumours are insufficient clinical indicators for correct diagnosis of the syndrome. Besides, the detection of inherited PCC/PGL's is important for patients with this syndrome, as well as for their family members, since they have increased risk of developing multiple and malignant neoplasms (Nederveen et al. 2009).

Mutation analysis of *SDHB*, *SDHC* and *SDHD* has been indicated to diagnose PCC/PGL's in all cases where there are no clear clinical or family signs for the syndrome. However, mutation analysis among PCC/PGL's patients frequently identify more cases without mutations than *SDH*-mutation carriers, making genetic screening approach a financially demanding and labour-intensive procedure (Nederveen et al. 2009).

Additionally, several reports have shown that a germline mutation in a *SDH* gene induces a complete absence of SDH enzyme activity in the tumour, irrespectively of the mutated SDH subunit - anchorage (SDHC and SDHD) or catalytic (SDHB) subunits -, suggesting a conformational change or a destabilization of the complex II (Gimenez-Roqueplo et al. 2001; Gimenez-Roqueplo et al. 2002; Dekker et al. 2003; Gill et al. 2010). In fact, there is an almost perfect correlation between the presence of a germline *SDHB*, *SDHC* or *SDHD* mutation and loss of SDHB expression on the tumour tissue, making SDHB immunohistochemistry the optimal method for discriminating between *SDH*-mutated and *SDH*-wild-type PCC/PGL's (Nederveen et al. 2009).

SDHB immunohistochemistry is highly sensitive and specific for the presence of an *SDH* mutation and is technically and financially reasonable, in particular in the absence of familial or clinical indications of a specific form of inherited PCC/PGL. Thus, genetic screening should only be performed when tumours show negative immunohistochemical SDHB expression. Obviously, this approach can only be performed whenever tumour tissue is available for study (Nederveen et al. 2009; Gill et al. 2010).

Additionally, *SDHA*-mutant tumours can be detected when the tumours are negative for SDHB and SDHA immunostaining, while *SDHB*, *SDHC* and *SDHD*-mutated tumours showed loss of SDHB expression and positive SDHA (Burnichon et al. 2010).

SDHD GENE

The *SDHD* gene is located on chromosome 11q23, has 4 exons and encodes a 103 amino acid protein. The SDHD protein is a small anchoring subunit implicated in electron transfer from iron sulphur clusters within SDHB, through an ubiquinone binding site (Fishbein et al. 2012). SDHD together with SDHC, form the anchoring structure of SDH in the mitochondrial complex II (Bardella et al. 2011).

PGL1 syndrome characterized by mutations in *SDHD* gene, has an autosomal dominant inheritance with a parent of origin effect. It is though that the gene is maternally imprinted, since the disease susceptibility occurs only when the mutation is inherited from the father, whereas no disease is observed when the mutation in inherited from the mother; however, the reasons behind this mode of inheritance are not yet fully understood. There is a rare case of a patient with jugulo-tympanic PGL showing a maternally transmitted *SDHD* mutation, but there is no available histological and molecular study of the tumour tissue to give more important information (Pigny et al. 2008; Bardella et al. 2011).

SDHD mutations can be missense, nonsense, frameshift and rarely splice-site (Benn et al. 2006).

Founder mutations have been discovered in different populations: American (p.Pro81Leu); Chinese (p.Met1Ile); Dutch (p.Asp92Tyr, p.Leu95Pro, p.Leu139Pro); Spanish (p.Trp43X); Italian (p.Gln109X) and Austrian (large Alu-mediated deletion – 4944 bp) (Bardella et al. 2011).

PGL1 appears by the age of 35, with a penetrance of about 86% by the age of 50 years. The parasympathetic PGL (often multifocal) is the most prevalent presentation (84%), followed by PCC (24%) and sympathetic PGL (22%). The risk of malignancy reaches only 5% (Welander et al. 2011; Fishbein et al. 2012).

SDHAF2 GENE

SDHAF2 is located on chromosome 11q12.2 and encodes a 167 amino acid protein which is involved in the incorporation of FAD cofactor in the subunit A of the succinate dehydrogenase complex, being essential for the SDH enzyme activity (Bardella et al. 2011; Fishbein et al. 2012; Jafri et al. 2012). Loss of SDHAF2 causes loss of SDHA function, decreasing the stability of SDH enzyme complex (Karasek et al. 2010).

PGL2 is associated with mutations in *SDHAF2* gene and is inherited in an autosomal dominant manner. Like PGL1, this syndrome is associated with a parent of origin effect, since disease susceptibility occurs only with paternal transmission of the mutation and the absence of disease occurs when mutation is maternally inherited (Welander et al. 2011).

The few cases reported to date with PGL2, describes early-onset tumours with the same founder mutation (Welander et al. 2011; Fishbein et al. 2012).

In a Dutch and Spanish family a germline loss-of-function mutation was identified in a conserved region of *SDHAF2* gene (p.Gly78Arg), resulting in decreasing flavination of SDHA subunits. Through the haplotype analysis of the two families it was discarded a common genetic origin between them, revealing that this mutation is a recurrent variant affecting the function of SDHAF2 protein (Baars et al. 1981; Bayley et al. 2010).

No other additional studies found mutations in *SDHAF2* gene, demonstrating that they are rare events (Bardella et al. 2011).

The mean age of PCC/PGL appearance is 33 years with a 100% penetrance by the age of 45 years (Welander et al. 2011; Fishbein et al. 2012). 91% of affected individuals have multifocal parasympathetic PGL's, with no malignancy associated (Fishbein et al. 2012). No cases of PCC's have been described in PGL2 syndrome (Karasek et al. 2010).

SDHC GENE

SDHC gene is located on chromosome 1q23.3, has 6 exons and encodes a 140 amino acid protein that consists of the large subunit cybL of cytochrome b in the mitochondrial complex II (Bardella et al. 2011; Fishbein et al. 2012).

Mutations in *SDHC* gene causes PGL3 syndrome with an autosomal dominant transmission (Fishbein et al. 2012). In contrast to PGL1 and PGL2 this familial form is not characterized by parent-of-origin effect since the disease occurs upon maternal and paternal transmission of the mutation (Bardella et al. 2011).

Mutations in this gene are less common than in *SDHD* and *SDHB*, occurring in 0 to 7% of PCC/PGL patients (Fishbein et al. 2012).

It was identified an Alu-mediated genomic deletion of 8.4kb involving exon 6 in a family and in an unrelated sporadic case with a common haplotype, supporting a common ancestral origin (Bardella et al. 2011).

The mean age of PCC/PGL onset is 38 years, ranging from 17 to 70 years old (Fishbein et al. 2012). Like in PGL1 and 2, parasympathetic PGL is the mainly tumour that develops in *SDHC* mutated patients (93%), with rare development of sympathetic PGL (7%) and more rarely PCC (Welander et al. 2011; Fishbein et al. 2012). The risk of malignancy is very low (Fishbein et al. 2012).

SDHB GENE

SDHB is located on chromosome 1p35-p36, has 8 exons and encodes a 280 amino acid protein that constitutes the iron sulphur subunit of the succinate dehydrogenase complex (Fishbein et al. 2012). This protein in combination with SDHA constitutes the catalytic domain of SDH (Bardella et al. 2011).

PGL4 syndrome is caused by *SDHB* mutations with an autosomal dominant inheritance and, like PGL3, both paternal and maternal disease transmission is observed (Bardella et al. 2011; Fishbein et al. 2012).

Point mutations, small insertions and deletions or duplications of the *SDHB* gene loci are examples of mutations that are associated with higher morbidity and mortality than mutations in other *SDH* genes (Welander et al. 2011; Fishbein et al. 2012).

Recently, *SDHB* missense mutations were associated with an increased risk of parasympathetic PGL when compared to truncating mutations. In addition, large deletions were also discovered in familial and apparently sporadic PCC/PGL cases that show phenotypes and penetrance similar to those of patients with point mutations. Large germline founder deletions were identified in individuals from Netherlands (7905 bp deletion in exon 3) and Spain (16 kb deletion involving exon 1). Two other mutations, p.P56delYfsX5 in Spanish population and IVS1 + 1 G>T in the Scottish population were also identified as having a possible founder effect (Bardella et al. 2011).

Most of the tumours associated with *SDHB* mutations are sympathetic PGL's in abdomen and pelvis (74%), but PCC's (25%) and parasympathetic PGL's (24%) are also observed (Fishbein et al. 2012). Tumours associated with *SDHB* mutations are often large, mainly solitary and with a strong tendency for metastasis (Karasek et al. 2010).

The mean age of PGL4 onset is about 33 years with a penetrance of 80-100% by the age of 70 (Welander et al. 2011; Fishbein et al. 2012). The risk of malignancy associated is substantial, ranging from 31 to 71%. This wide range of malignancy risk is associated with long time to develop metastases, which is thought to be up to 20 years after primary tumour diagnosis (Fishbein et al. 2012). Because of the high potential of malignancy and poor prognosis, *SDHB* carriers should start screening of PGL/PCC at the age of 10 (Bardella et al. 2011).

Mutations in *SDHB* are associated with the development of other neoplasias such as gastrointestinal stromal tumours (GIST), breast and papillary thyroid carcinoma (PTC), neuroblastoma and renal cell carcinoma (clear-cell and papillary RCC) (Karasek et al. 2010; Fishbein et al. 2012).

PCC/PGL's related with *SDHB* and *SDHD* have a biochemical phenotype that allows to distinguish them from tumours associated with *VHL*, *RET* or *NF1* mutations. PGL1 and PGL4 patients have an increased level of methoxytyramine which indicates dopamine hypersecretion that can occur alone or in association with norepinephrine hypersecretion (*SDHB*-related tumours) (Karasek et al. 2010).

SDHA GENE

SDHA gene is located on chromosome 5p15 and encodes a 621 amino acid protein – flavoprotein – which is the major catalytic subunit in the succinate dehydrogenase complex, comprising a prosthetic group covalently attached to FAD (Bardella et al. 2011; Fishbein et al. 2012). This subunit binds enzyme substrates - succinate and fumarate – as well as physiological regulators – oxaloacetate and ATP (Bardella et al. 2011).

Recently, Burnichon et al. discovered, in a patient with abdominal PGL, the first heterozygous germline missense mutation (p.Arg589Trp) associated with somatic LOH at the *SDHA* locus within the tumour (Burnichon et al. 2010). Subsequently, the same group reported a series demonstrating that *SDHA* mutations represent 3% of germline mutations in apparently sporadic PCC/PGL (Korpershoek et al. 2011). So far, only six patients with PCC/PGL's were identified with *SDHA* mutations, three with sympathetic PGL, two with parasympathetic PGL and one of them with PCC (Welander et al. 2011; Korpershoek et al. 2012).

Interestingly, *SDHA* germline homozygous mutations that cause loss or reduced enzymatic activity are associated with other diseases like Leigh's syndrome (encephalopathy), a neurodegenerative disorder with an early onset and autosomal recessively inheritance, as well as optic atrophy, ataxia and myopathy, late-onset disorders (Bardella et al. 2011; Fishbein et al. 2012; Jafri et al. 2012).

As the number of mutations in *SDHA* gene is very low, the prevalence of mutations and risk of malignancy are not yet determined (Fishbein et al. 2012).

The mean age of *SDHA* related PCC/PGL's development is about 40 years old (Fishbein et al. 2012).

TMEM127 GENE

TMEM127 - transmembrane protein 127 - is located on chromosome 2q11.2, has 4 exons (the first is non-coding) and encodes a 238 amino acid protein. TMEM127 protein comprises three transmembrane domains and co-localizes with the early endosome in the cell, being involved in the mTORC1 (mechanistic target of rapamycin complex 1) signalling pathway (Fishbein et al. 2012).

In 2005, *TMEM127* was recognized as a PCC/PGL's susceptibility gene after the identification of germline loss-of-function mutations, particularly missense and nonsense, in patients with PGL and PCC. It functions as a tumour suppressor gene with presence of LOH identified in all tumours carrying a mutation (Dahia et al. 2005).

Mutations in this gene have a frequency of about 2% and confer tumour susceptibility in an autosomal dominant pattern (Fishbein et al. 2012; Jafri et al. 2012).

Most of the tumours associated with *TMEM127* mutations are PCC's (96%) with 39% being bilateral PCC's, followed by PGL's (9%) with an equal frequency between sympathetic and parasympathetic PGL's (Welander et al. 2011). The mean age of onset is 45 years and the malignancy risk is very low (Fishbein et al. 2012).

Other tumours are associated with mutations in *TMEM127* gene, such as medullary thyroid carcinoma, breast cancer and myelodysplasia, but the causal link is still under debate (Welander et al. 2011).

TMEM127 is particularly localized in multiple endomembrane organelles such as endosomes, Golgi apparatus and lysosomes and is associated with endosomal pools, participating in protein trafficking between the plasma membrane and these organelles. mTOR components are also confined to endosomal and vacuolar compartments (Jiang et al. 2011).

The function of TMEM127 is not well established, but it is thought that it functions as a negative regulator of mTOR (Welander et al. 2011).

Loss of function *TMEM127* mutations leads to increased phosphorylation of mTORC1 targets such as 4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) and S6K (ribosomal protein S6 kinase), as well as increased cell size and proliferation in numerous cell lines. *In vivo* studies showed the same hyperphosphorylation of mTORC1 targets in patients with *TMEM127* mutations and no changes in phosphorylation of mTORC2 targets, suggesting that TMEM127 is a negative regulator of mTORC1 but not mTORC2. Despite the aforementioned findings, the precise role of TMEM127 in mTOR pathway is still to be uncovered. In fact, TMEM127 is considered a member of a newly "oncogenic endomembrane network", a new regulatory system in cancer whose functions are still unknown (Jiang et al. 2011).

MAX GENE

MAX gene or myc-associated factor X is located on chromosome 14q23, has five exons and encodes a transcription factor with 160 amino acids, being a member of the basic helix-loop-helix leucine zipper family that comprises the MYC, MAX and MAD proteins (Welander et al. 2011; Fishbein et al. 2012; Jafri et al. 2012).

The MYC family proteins, c-MYC, n-MYC and l-MYC are oncoproteins associated with cell proliferation, differentiation and neoplasia (Ecevit et al. 2010).

MAX is similar to MYC, but lacks the transactivation domain (Ecevit et al. 2010). All known oncogene functions of MYC require that MAX heterodimerizes with MYC in the MYC-MAX-MAD network, resulting in transcription activation of target genes through the *trans*-activating domain of MYC which is associated with cellular proliferation, differentiation and apoptosis. On the other hand, when MAX heterodimerizes with MAD, the transcription repression of the same target genes occurs, a function that antagonizes MYC-MAX heterodimer (Ecevit et al. 2010; Welander et al. 2011; Fishbein et al. 2012; Jafri et al. 2012). Therefore, the binding of these similar transcription factor dimers to the E-box determines the cell destiny: division and proliferation with MYC-MAX dimer or differentiation and quiescence with MAD-MAX dimer (Ecevit et al. 2010).

MAX was identified as a susceptibility gene for PCC/PGL by Comino-Méndez et al. using whole exome sequencing on germline DNA (Comino-Méndez et al. 2011). The mutations identified were

inactivating mutations particularly missense, nonsense and splice site; the concomitant LOH and loss of MAX expression in the tumour tissue are consistent with tumour suppressor function (Welander et al. 2011; Jafri et al. 2012).

Similarly to what happens in PGL1 and PGL2 syndromes, *MAX* mutations have a parent-of-origin effect since paternal transmission of the gene is necessary for tumour development (Jafri et al. 2012).

Patients with *MAX* mutations exhibit bilateral PCC's in 67%; no studies on large series of PGL's were performed so far. The mean age at presentation is about 32 years and the malignancy risk is high (Welander et al. 2011).

OTHER GENES

RET GENE

RET - rearranged during Transfection - gene was discovered in 1985 by transfection of NIH 3T3 cells with human lymphoma DNA (Welander et al. 2011). *RET* is located on chromosome 10q11.2, has 21 exons and encodes an 860 amino acid transmembrane receptor tyrosine kinase whose main function is the regulation of cell proliferation and apoptosis (Fishbein et al. 2012). When a specific ligand, particularly the members of the glial cell line-derived neurotropic factor family (GDNF), bind to RET receptor, a dimerization is induced and triggers phosphorylation of specific tyrosine residues and activation of multiple intracellular pathways (Karasek et al. 2010; Welander et al. 2011; Fishbein et al. 2012).

The RET protein acts in the development of the kidney and sympathetic, parasympathetic and enteric nervous system, thus it is mostly expressed in urogenital and neural crest precursor cells (Welander et al. 2011).

Germline gain of function *RET* mutations predispose to multiple endocrine neoplasia type 2 (MEN2), which is an autosomal dominant syndrome with a prevalence of 1/40 000 individuals (Karasek et al. 2010; Welander et al. 2011).

Over 50 different missense mutations have been identified in MEN2 families and can be found in exon 10 codons 609, 611, 618, 620 and exon 11 specifically in codons 630, 634, all of them resulting in cysteine substitution; exon 13 codons 768 (Glu→Asp), 790 (Leu→Phe) and 791 (Tyr→Phe); exon 14 codon 804 (Val→Leu); exon 15 codons 883 (Ala→Thr) and 891 (Ser→Ala) and exon 16 codon 918 (Met→Thr). PCC/PGL development is associated with codons 634 and 918 mutations in approximately 50% of patients, with exon 10 mutations in up to 20% patients, and rarely with mutations in exons 13 to 15 (Benn et al. 2006; Quayle et al. 2007; Raue et al. 2010; Welander et al. 2011).

PCC/PGL's related with MEN2 overexpress phenylethanolamine N-methyltransferase, an enzyme that converts norepinephrine to epinephrine. Consequently, the levels of epinephrine are increased, resulting in PCC symptoms – anxiety, headaches, and palpitations. Another feature of this familial syndrome is the presence of increased amounts of metanephrine, a metabolite of epinephrine, in plasma and urine, making possible a distinguishable pattern from patients with *VHL* and *SDHx* mutations (Karasek et al. 2010).

MEN2 can be divided into three subgroups:

- MEN2A (55% of all cases): 95% of the patients have medullary thyroid carcinoma (MTC), 50% have PCC and 15-30% have hyperparathyroidism caused by parathyroid hyperplasia or adenoma; the majority of patients have a mutation in codon 634 in exon 11 of *RET* (p.Cys634Arg or p.Cys634Tyr) (Karasek et al. 2010; Welander et al. 2011; Fishbein et al. 2012; Jafri et al. 2012).
- MEN2B (5-10% of all cases): 100% of the cases have MTC, 50% have PCC, marfanoid habitus and multiple mucosal ganglioneuromas; it is the most aggressive form of MTC with higher morbidity and mortality and earlier onset; a single missense mutation in codon 918 in exon 16 (p.Met918Thr) is the principal mutation (Karasek et al. 2010; Welander et al. 2011; Fishbein et al. 2012; Jafri et al. 2012).
- Familial medullary thyroid carcinoma (35-40% of all cases): patients have only MTC, often less aggressive; mutations in the codons 768, 790, 804 and 891 are associated with only MTC susceptibility (Karasek et al. 2010; Welander et al. 2011; Fishbein et al. 2012).

RET mutations predispose to PCC that often presents as recurrent, bilateral (50% of patients) and with low risk of malignancy (5%); in contrast, PGL's are very rare in MEN2 syndrome. The age of onset of MEN2 related PCC is commonly between the ages of 30 and 40 years (Karasek et al. 2010; Welander et al. 2011).

VHL GENE

VHL tumour suppressor gene is located on chromosome 3p25-26 and has 3 exons. More than 300 mutations have been identified such as missense, nonsense, deletions and insertions (indels) that cause the Von Hippel-Lindau syndrome which is characterized by the development of both benign and malignant tumours in numerous organs. These are heterozygous germline mutations often associated with somatic loss of heterozygosity (LOH) of the wild-type allele in the tumour tissue (Benn et al. 2006; Karasek et al. 2010; Welander et al. 2011). About 20% of families have *de novo* mutations (Karasek et al. 2010).

VHL protein function is to regulate the hypoxia inducible factor-1 alpha (HIF-1α) activity in blood vessel formation (Karasek et al. 2010). VHL protein has E3 ubiquitin ligase activity, which ubiquitinates HIF-1α that consequently undergoes proteasomal degradation, under normoxic conditions (Welander et al. 2011; Fishbein et al. 2012). In hypoxic conditions or when there is a mutation in *VHL*, HIF-1α is no longer degraded, being able to target the transcription of hypoxia inducible genes after complexing with HIF-1β and other proteins (Fishbein et al. 2012).

Von Hippel-Lindau (VHL) is an autosomal dominant syndrome with a prevalence of 1/36 000 individuals that can be divided into two types (Karasek et al. 2010):

- VHL type 1: it is the most common form, without predisposition to develop PCC and associated with clear-cell renal carcinomas, retinal angiomas, central nervous system hemangioblastomas (brain, spinal cord and retina), islet tumours of the pancreas, endolymphatic sac tumours, cysts and cystadenomas (kidney, pancreas, epididymis and broad ligament); the most frequent mutations are truncating mutations or exon deletions (Karasek et al. 2010; Fishbein et al. 2012).

- VHL type 2: it is associated with PCC development and associated with missense mutations; the tumour spectrum of VHL type 2 depends on the location of the mutation (Karasek et al. 2010; Fishbein et al. 2012). This type can be divided in three subtypes:
    - VHL type 2A: without renal carcinoma and less predisposition to VHL type 1 tumours (Karasek et al. 2010).
    - VHL type 2B: includes all VHL type 1 tumours (Karasek et al. 2010).
    - VHL type 2C: only PCC development (Karasek et al. 2010).

Considering all subtypes of VHL, PCC/PGL development occurs in 10-26% of patients, with PCC's as the most commonly form of catecholamine producing tumours (90%), although sympathetic and parasympathetic PGL tumours have been identified in rare occasions (19%) (Karasek et al. 2010; Welander et al. 2011). PCC presents as bilateral in more than 50% of the cases and is less malignant than sporadic PCC (<5% of patients) with a mean age of presentation of 30 years (Karasek et al. 2010). In families with PCC/PGL, missense mutations affecting the surface of the folded protein are more frequent than those affecting the deeper protein core or the ones that disrupt the interaction between VHL and HIF (associated with clear-cell renal carcinoma) (Welander et al. 2011; Fishbein et al. 2012).

VHL patients present increased plasma and urinary levels of normetanephrine, which occurs due to low expression of pehnylethanolamine-N-methyltransferase with a consequently decreased production of epinephrine and increased production of norepinephrine (Karasek et al. 2010). VHL-associated PCC/PGL's are frequently asymptomatic, comparing to sporadic PCC/PGL's (Jafri et al. 2012).

NF1 GENE

NF1 gene was discovered in 1990 and is a tumour suppressor gene with over 60 exons, located on chromosome 17q11.2 that encodes a 2818 amino acids protein, named neurofibromin. This protein is particularly expressed in the nervous system and its main function is the GTPase activity to convert Ras into its inactive form, thereby inhibiting Ras/Raf/MAPK signalling pathway, as well as PI3K/AKT/mTOR pathway (Welander et al. 2011; Fishbein et al. 2012). When a *NF1* mutation is

present, the mutant protein does not inactivate Ras, which becomes constitutively activated to trigger the subsequent oncogenic downstream pathways – MAPK, PI3K and mTOR (Fishbein et al. 2012).

Neurofibromatosis type 1 (NF1), also termed von recklinghausen's disease is an autosomal dominant genetic disorder with an incidence of 1/3000 persons (Fishbein et al. 2012; Jafri et al. 2012).

The alterations in *NF1* gene comprise inactivating mutations such as missense, nonsense, splice-site mutations and indels (Welander et al. 2011).

Thirty to 50% of NF1 patients have mainly *de novo* mutations that can give rise to a mosaic phenotype (Welander et al. 2011; Fishbein et al. 2012). There is no hot spot in this large gene making it difficult to perform a genetic screening of the disease (Fishbein et al. 2012). As such, the diagnosis is based on clinical criteria that consist in having at least two features of the following ones (Karasek et al. 2010; Fishbein et al. 2012):

- two or more cutaneous neurofibromas or a single plexiform neurofibroma;
- two or more Lisch nodules (benign iris hamartomas);
- six or more café-au-lait macules with specific size depending on age;
- inguinal or axillary freckling;
- at least one optic nerve glioma;
- dysplasia of the long bones or pseudoarthrosis;
- first-degree relative with NF1.

Several tumours have been identified with high frequencies in NF1 patients, such as medullary thyroid carcinoma, parathyroid tumours, carcinoid tumours of the duodenal wall, peripheral nerve sheath tumours and leukemia (mainly chronic myeloid leukemia) (Karasek et al. 2010; Fishbein et al. 2012).

PCC/PGL occurs in NF1 syndromes with an estimated rate of 0-6% (up to 13% at autopsy) and 20-50% in NF1 patients with hypertension (Karasek et al. 2010; Welander et al. 2011). Most of them are unilateral PCC's (95%) and rarely bilaterally PCC's (14%) or sympathetic PGL's (6%) (Welander et al.

2011). The potential malignancy is around 12%. The mean age at presentation of PCC is 42 years (Karasek et al. 2010; Welander et al. 2011; Fishbein et al. 2012).

The main alterations occurring in PCC's associated with NF1 patients comprise changes in both alleles including one germline mutation and one acquired mutation or LOH of the wild-type allele (Welander et al. 2011).

PCC related with NF1 syndrome has overproduction of epinephrine that causes an increase of metanephrine in the plasma and urinary levels (Karasek et al. 2010).

OTHER SYNDROMES WITH RELATED PCC/PGL

CARNEY'S TRIAD SYNDROME

Carney's Triad is a disorder that comprises PCC/PGL's, GIST's and pulmonary chondromas. This syndrome affects young women with a mean age of presentation of 21 years. Most of the patients with this disease have only two of the three tumours, mainly GIST and pulmonary chondroma; about 20% have the all three tumour types of the triad (Welander et al. 2011).

So far, no mutations in genes involved in familial PGL's (*SDHA, SDHB, SDHC, SDHD*) or GIST (*KIT* and *PDGFRA*) were associated with Carney's Triad syndrome (Welander et al. 2011).

PGL's appear in 92% of Carney's Triad patients, both sympathetic and parasympathetic, and PCC's are present in 16%. The mean age at presentation of PCC/PGL's in this syndrome is 28 years and metastasis occurs in about 11% of patients (Welander et al. 2011).

CARNEY-STRATAKIS SYNDROME

Carney-Stratakis or Carney's Dyad includes two types of tumours: PCC/PGL's and GIST's. It is transmitted by an autosomal dominant manner with incomplete penetrance. The mean age at presentation of the syndrome is 23 years with similar incidence in men and women. Most of the

patients display only PGL/PCC's (58%), followed by patients with both tumours (33%) and a minority with only GIST (8%) (Welander et al. 2011).

In contrast to Carney triad, the Carney dyad patients carry germline mutations in *SDHB*, *SDHC* or *SDHD* genes (Welander et al. 2011).

In this syndrome, PGL's appear in almost all the patients with only 9% frequency for PCC's. The mean age at presentation is 33 years and with no malignancy associated (Welander et al. 2011).

SPORADIC PCC/PGL'S

The majority of PCC/PGL's cases present as sporadic cases, without known familial history. Nevertheless, inherited mutations are present in 11 to 24% of patients with negative familial history of disease (Welander et al. 2011).

Patients with apparently sporadic tumours have an older onset with a mean age at presentation of 48 years and lower frequency of multiple tumours comparing to those with familial history (Welander et al. 2011).

The majority of sporadic cases are PCC's, 73%, with only 20% of parasympathetic PGL's, 9% of sympathetic PGL's and 6% of bilateral PCC's; the rate of malignant disease is low (9%) in these presentations (Welander et al. 2011).

GENETIC TESTING APPROACHES

Considering the large number of genes involved in PCC/PGL, it is important to establish criteria to manage the genetic screening. The probability of detecting a mutation in an individual case includes the following clinical features (Jafri et al. 2012):

- a personal or family history that indicates a syndromic cause, for example MTC in MEN2 syndromes or hemangioblastoma in VHL syndrome;

- a positive family history for PCC;
- multiple primary PCC's;
- malignancy;
- extra-adrenal location;
- young age at diagnosis.

Therefore, the personal history, family history and clinical examination are the major criteria for assessment of an appropriate germline mutation. In case of having a family history or evidence of specific features of each familial syndrome, targeted genetic testing should be done. Predictive testing allows the identification of asymptomatic patients who are at risk for disease development, which consequently will help in biochemical and radiologic screening, reducing morbidity and mortality (Karasek et al. 2010).

Patients with a positive familial history have 90% probability of having a specific gene mutation. Germline mutations in non-syndromic patients is estimated about 18-19%, but in the case of multiple or recurrent PCC/PGL's, the frequency is about 39% (Karasek et al. 2010).

Sporadic PCC/PGL's without familial history or other symptoms of familial syndromes show genetic mutations in 18-19% of the cases. In multiple or recurrent PCC/PGL's the frequency of mutations is about 39% (Karasek et al. 2010).

In patients with early onset, bilateral, multifocal, extra-adrenal, tumours with recurrent or malignant disease, a germline mutation is likely to be present (Karasek et al. 2010).

NF1 is identified by a careful physical examination, discarding a genetic testing for *NF1* gene (Jafri et al. 2012).

For the other genes, a genetic testing diagram can be followed in the diagnosis (Figure 3).

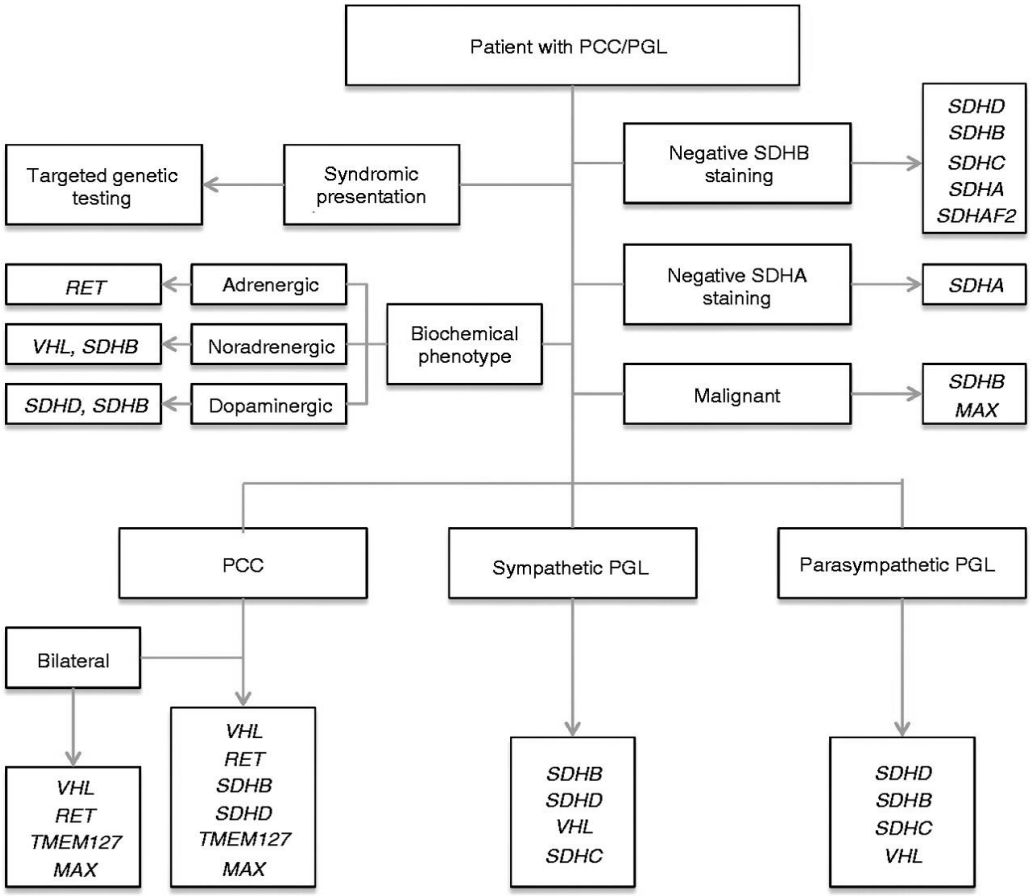


Figure 3 – Diagram of the genetic testing protocol as an example of diagnostic approach (Welander et al. 2011).

If the patient has a positive family history, syndromic lesions or both, the appropriate specific gene test should be performed. In non-syndromic patients or without family history, immunohistochemical analysis for SDHB should be done to identify *SDH*-related tumours in negative SDHB staining. If it is the case, *SDH* genetic analysis is the second step to find mutations that lead to loss of SDHB expression. If the immunohistochemistry shows positive SDHB expression, other genes should be tested, such as the *MAX* and *TMEM127* genes, according to type of tumour (Karasek et al. 2010).

Furthermore, since *MAX* mutations demonstrated loss of MAX expression in the tumour tissue, immunohistochemistry for MAX can be considered as a potential marker for *MAX* germline mutations (Jafri et al. 2012).

3. AIMS

It is currently known that a significant proportion of PCC/PGL's is due to germline mutations in ten susceptibility genes – *SDHA, SDHB, SDHC, SDHD, SDHAF2, MAX, VHL, TMEM127, RET* and *NF1*. The main aim of this work was to perform a molecular analysis of blood and tumour samples in patients with PCC/PGL's, in order to establish the molecular basis of the disease. For that matter, we have looked for mutations in *SDH*, *SDHAF2*, *MAX* and *TMEM127* genes in patients without evidence of syndromic (MEN2, VHL or NF1) disease; whenever a patient demonstrated signs of such syndromes, the causative gene was studied. Once identified the genetic alterations, we looked for genotype-phenotype correlations in an attempt to progress in the clinical management of PCC/PGL patients.

From the studies reported so far, the prevalence of mutations in the aforementioned ten genes is associated with the population under study. Here, we had access to Polish and Portuguese populations with PCC/PGL's, allowing a similar study to those performed for other populations. This thesis is divided taking into account these two different populations: the first part concerning a Polish population with only PCC's; the second part regarding Portuguese population with PCC or PGL.

PART I

The first part of the thesis comprised a series of 61 samples of paraffin embedded tissue from Polish PCC patients. This series had already been clinically characterized and we had contact with a pathologist from Poland responsible for these cases, which provided us all the clinico-pathological information collected from the patients.

Thus, based on previous reports about PCC/PGL's, we decided to perform an expression study for SDH protein followed by a genetic screening for *SDHB, SDHC, SDHD, SDHAF2, TMEM127* and *MAX*.

More specifically, an immunohistochemical study in order to find tumours with loss of SDH enzyme expression was performed; the samples with loss of expression were screened for mutations in *SDH* (except *SDHA*) and *SDHAF2* genes to find the pathogenic mutation responsible for PCC susceptibility. In those tumours with presence of SDHB expression (indicating absence of *SDH* mutations), genetic characterization for *MAX* and *TMEM127* genes was accomplished. Finally,

genotype-phenotype correlations were accomplished in order to take some conclusions about this population of PCC's.

PART II

Concomitantly, in the second part of this thesis, 12 blood samples from Portuguese PGL/PCC patients were analysed. In this series no tumour tissue was available to perform the immunohistochemistry, so the approach was to screen for mutations in *SDH* genes (*SDHB, SDHC, SDHD*). Like in the Polish population, genotype-phenotype correlation was done to draw conclusions about the Portuguese population of PCC/PGL's.

4. MATERIAL AND METHODS

PART I

PATIENTS AND CASES

The cases included in the first part of the study comprised paraffin-embedded tissue from 61 PCC tumours belonging to 57 Polish patients. Personal and clinico-pathological information was provided by a pathologist including patient's age and sex, as well as tumour location, size, histological features and microvessel density.

Among the 57 patients diagnosed with PCC, 50 cases had no family history (88%) and the remaining 7 PCC's were associated with familial syndromes (12%); in the latter, two patients presented with MEN2 (29%), four with NF1 (57%) and one with VHL syndrome (14%).

Metastatic disease in PCC is defined by the presence of tumour cells in sites that normally do not contain chromaffin cells (Thompson 2002; Fishbein et al. 2012). Malignancy is usually identified using histologic observations of PCC, like increased necrosis, vascular and capsular invasion (defined by direct extension into the vessel lumen, intravascular attached tumour thrombi and/or tumour nests covered by endothelium in a capsular or extracapsular vessel), presence of mitotic figures (number of mitoses per 10 high power fields – HPF) and atypical mitotic figures, periadrenal adipose tissue infiltration, diffuse growth, high cellularity, presence of spindle cells, cellular monotony, cellular pleomorphism and nuclear hyperchromatosis (Jafri et al. 2012). Additionally, a Pheochromocytoma of the Adrenal Gland Scaled Score (PASS) is used for these specific histologic features and separates tumours with potential aggressive behaviour (PASS≥4) from biologically benign tumours (PASS<4), helping to predict prognosis. Thus, each feature accounts for a specific score if present in the tumour, as revealed in Table 1, which result in a final PASS score (1-20) for the PCC and allows the identification of benign or malignant one (Thompson 2002).

Table 1 – Pheochromocytoma of the Adrenal Gland Scoring Scale (PASS) (Thompson 2002).

Histologic feature	Score (if present)
Diffuse growth	2
Single cell necrosis	2
High cellularity	2
Cellular monotony	2
Spindle cells	2
More than 3 mitotic figures/10HPF	2
Atypical mitotic figures	2
Periadrenal adipose tissue infiltration	2
Vascular invasion	1
Capsular invasion	1
Cellular pleomorphism	1
Nuclear hyperchromatosis	1
Total	20

HPF – high power fields

Microvessel density was also assessed in every tumour and scored using the Chalkley counting method. The Chalkley score was applied to count vessels under the capsule and in the middle of the tumour using mm^2 as counting area.

In this method tissue sections were stained for tumour vasculature and a Chalkley graticule was applied. Chalkley graticule is a circle with 25 dots randomly placed in a vascular area and the greatest number of vessels that coincide with this graticule is averaged for the three hotspot areas to define the Chalkley score (Jubb et al. 2006; Hayat 2010).

This method uses antibodies against vascular endothelium for the assessment of tumour vascularity, detecting microvessel density (Hayat 2010).

Tumour microvessel density may predict the risk of metastasis and serum levels of angiogenic factors may serve as tumour markers (Ranieri et al. 2001). Thus, different endothelial cell markers are used to evaluate microvessel density in tumours, such as anti-CD31 and anti-CD105 selected for this study (Hayat 2010) .

CD31 also known as PECAM-1 – platelet endothelial cell adhesion molecule-1 – is a membrane protein with 130 kDa and belongs to the immunoglobulin superfamily whose function is mediating cell-to-cell adhesion. This protein is expressed in the surface of adult and embryonic endothelial cells, bone marrow-derived hematopoietic stem cells, embryonic stem cells and weakly in peripheral leukocytes and platelets. CD31 is also involved in angiogenesis as mediator of endothelial cell-cell interactions (Vecchi 1994).

CD105, known as endoglin is an endothelial membrane antigen expressed on the endothelial cells of capillaries, arterioles and venules as well as strongly expressed in blood vessels of tumour tissues. This glycoprotein is part of the transforming growth factor receptor complex whose function is to be receptor for TGFβ 1 and 3 and modulate TGFβ by interacting with TGFβ receptors I and II (Duff et al. 2003).

Specifically for the assessment of microvessel density, CD31 shows the vascular status of the tumour but they mark both neoformed vessels and normal preexistent vessels in normal and tumoural tissues, not indicating the angiogenic intensity; CD105 seems to be more specifically for the endothelial cells of neoformed vessels, as its expression increased with the angiogenic progression (Jung et al. 2009).

METHODS

IMMUNOHISTOCHEMISTRY

Histopathology of the tumours was evaluated using 2µm sections and conventional Haematoxylin and Eosin (H&E) staining.

The immunohistochemistry staining of SDHB and SDHA proteins in PCC tumour tissues was executed by Thermo Scientific/Lab Vision kit (Fremont, USA).

Paraffin was dissolved by Clear Rite solution, followed by a series of ethanol's (100%, 95% and 70%) and running water that permeabilize and hydrate the tissues. Antigenic retrieval was performed using 1xEpitope Retrieval Solution pH 9 (Tris/EDTA-based buffer containing surfactant) (Leica

Microsystems, Newcastle Upon Tyne, United Kingdom) at 98°C for 20 min in water bath for SDHB and 1 min at 98°C followed by 5 min at 125°C, in a pressure cooker for SDHA. Next, a series of blocking solutions were added: 3% hydrogen peroxide in methanol to block the endogenous peroxidase activity; Avidin-Block and Biotin-Block solutions (Thermo Scientific/Lab Vision, Fremont, USA) for endogenous biotin blockage and Large Volume Ultra V-Block (Thermo Scientific/Lab Vision, Fremont, USA) for non-specific binding blockage.

Incubation with primary antibody was performed using mouse monoclonal antibodies specific for SDHA (Complex II subunit 70 kDaFp) and SDHB (Complex II subunit 30 kDaIp) from MitoSciences (Eugene, Oregon, USA). Negative (without primary antibody) and positive controls were used simultaneously to ensure specificity and reliability of the staining process. The antibodies were diluted in "Large Volume Ultra Ab Diluent" (Thermo Scientific/Lab Vision, Fremont, USA) using the exact dilution for each one and incubated for a specific time and temperature: SDHA – 1:1250, 1h at room temperature; SDHB – 1:600, overnight at 4°C (Table 2).

Antigen detection was done with a labelled streptavidin-biotin immunoperoxidase detection system (Thermo Scientific/Lab Vision, Fremont, USA) using a secondary antibody "Biotinylated Goat Anti-Polivalent" and a streptavidin-peroxidase complex "Large volume Streptavidin Peroxidase". Finally, an immunohistochemical staining with DAB chromogen (3,3'-diaminobenzidine) (Thermo Scientific/Lab Vision, Fremont, USA) was used followed by hematoxylin as counterstain to visualize the presence or absence of antibody-antigen binding.

Table 2 – Immunohistochemistry for SDHB and SDHA.

Protein	Antigenic Retrieval	Antibody		
		Name	Dilution	Incubation
SDHB	Water Bath 20 min 98°C	Complex II subunit 30 kDa (MitoSciences)	1:600	ON 4°C
SDHA	Pressure cooker 1min 98°C 5min 125°C	Complex II subunit 70 kDa (MitoSciences)	1:1250	1h RT

DNA EXTRACTION

Slides with 10µm sections were immersed in Clear Rite to dissolve paraffin and then in 100% ethanol to wash the tissue. Next, tumour and normal tissue was microdisseced into different eppendorfs. Cell lysis solution (Citomed, Lisbon, Portugal) and 180µg/ml proteinase K (Fermentas, Thermo Fisher Scientific) was added to each sample in order to digest the cells at 55°C overnight. After lysis and digestion, a protein precipitation solution (Citomed, Lisbon, Portugal) was added to each eppendorf and, after centrifugation (16000g, 3min., 4°C), supernatant was transferred to another eppendorf. In the new eppendorfs, DNA was precipitated after adding isopropanol (1x volume) and 10µg/ml glycogen (Fermentas, Thermo Fisher Scientific). After centrifugation (3x 16000g, 10min., 4°C), the DNA pellet was washed with 70% ethanol and finally eluted in deionized water and quantified in NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Fremont, USA).

PCR – POLYMERASE CHAIN REACTION

Polymerase Chain Reaction was used to amplify the exon and exon boundaries of the genes of interest. The Qiagen® Multiplex PCR kit was used, according to manufacturer's instructions. Amplifications were performed using 2x Qiagen Multiplex Master Mix (10µL), 5x Q-Solution (2µL), 1x forward and reverse primers (1µL) and template DNA (10-100 ng/µL) in a 20 µL reaction volume.

Cases with familial history of MEN2 and VHL syndrome, were screened for mutations in *RET* and *VHL* genes, respectively. Cases showing a negative immunostaining for SDHB protein were screened for mutations in *SDH* genes (except *SDHA* gene), beginning with a recently found large deletion in *SDHB* gene comprising promoter and exon 1 with Portuguese origin. All samples presenting this deletion were then tested for the haplotypic variability in the flanking regions upstream and downstream of the deletion breakpoint, already identified among the previous cases showing this mutation, in order to demonstrate the same haplotype of all cases. When the deletion was not present, *SDHB* gene was tested for other mutations, followed by *SDHD* and finally *SDHC* genes as the most suitable procedure. Finally, cases without SDH, MEN2, NF1 and VHL syndrome associated were screened for mutations in *TMEM127* and *MAX* genes.

Primers used for *SDHB* large deletion, haplotype analysis and *RET* (exons 11 and 16)*, VHL*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *MAX* and *TMEM127* genes are listed in Table 3.

Six independent multiplex reactions were performed for simultaneous amplification of exons 1-4 of *SDHD*, exons 1-4 and 5-8 of *SDHB*, exons 1-3 and 4-6 of *SDHC* and exons 1-4 of *SDHAF2*.

For *MAX* and *TMEM127*, exons were amplified individually using the same Multiplex PCR kit (exon 1 of *TMEM127* was not amplified since it is a non-coding exon).

The mixture was heated for 15 minutes at 95°C for initial DNA denaturation, followed by 35 or 40 cycles (depending on the tissue quality) of denaturation (30 sec at 95°C), annealing (90 sec at the following temperatures: *RET* exon 16 at 55°C; *SDHB* at 60°C; *SDHC* and *SDHAF2* at 58°C; *SDHD* at 56°C; *MAX* exons 1 and 3 at 65°C, exons 2 and 4 at 61,1°C and exon 5 at 58,7°C; *TMEM127* exons 2 and 4 at 63°C and exon 3 at 58,7°C) and extension (90 sec at 72°C); the last step is the final extension at 72°C for 10 min. Annealing for *RET* exon 11 and *VHL* was done by touchdown in cycles of 90 sec, beginning with 64°C for 2 cycles, 62°C for 3 cycles and 60°C for 35 cycles in the *RET* gene; 62°C for 2 cycles, 60°C for 3 cycles and 58°C for 35 cycles in the *VHL* gene.

Table 3 – Primers sequences for *SDHB* large deletion (promoter and exon 1) and haplotype; *VHL*, *RET* (exon 11 and 16), *SDHB, SDHC, SDHD, SDHAF2, MAX* and *TMEM127* genes.

Primer name		Primer sequence
SDHB Deletion promoter+exon 1	5UPUP StreamF	AGCGCCAATTGTGGAAATAG
	BREAK R	GCCTGAGGCAGATAGTAGGG
Haplotype	5UPUP StreamF	AGCGCCAATTGTGGAAATAG
	Intron 1.2R	CCCTACCTCCCCTGTACTCC

Primer name		Primer sequence
VHL	1F	TGGTCTGGATCGCGGAGG
	1R	CTAAGCGCCGGGCCCGT
	2F	ACAACCTTTGCTTGTCCCGA
	2R	AGTGGTCTATCCTGTACTTACCA
	3F	ACCCTAGTCTGCCACTGAGGA
	3R	AGCTGAGATGAAACAGTGTAAGT
***RET* hotspots**	11F	CCCAGTGGTGCCGAGCCTCT
	11R	GTGGATGCAGAAGGCAGACAGC
	16F	CCTCCTTCCTAGAGAGTTAGAGT
	16R	AAGAGAGCAACACCCACACT
SDHB	1F	GGTCCTCAGTGGATGTAGGC
	1R	ACTTTTCCCTCTCTGAGGCT
	2F	GCGTTACATCTGTTGTGCCA
	2R	AGCTGGCTTTCACAGAGATAC
	3F	ACATCCAGGTGTCTCCGATT
	3R	AGCCCAACAGGAATGAAATG
	4F	TGATTCCGGATATGGGTGAG
	4R	ACCAGAGAGATGCAGAAACTC
	5F	GCTGAGGTGATGATGGAATCT
	5R	AGCCACACTCCTGGCAATCA
	6F	ATAGCAGAGTCTCTCCCGTC
	6R	AGCAATTAAGGAGCACCTCA
	7F	CTCAGCTAATCATCCCTGGT
	7R	TCTGGCGTGTCAGCTCTGA
	8F	AGGAAGGAGTTTCACCCAAG
	8R	ATGTTCAGCTCTGAGCTGGT
SDHC	1F	ACTTCCGTCCAGACCGGAA
	1R	AACGTGAGGGGCCAGTTCA
	2F	AGTTGATCTCTAAATGTGTATTGA
	2R	AATAATCTCCAGACTTAGAAACTT
	3F	ATGCCTGGCTTGGTATTGCAA
	3R	ATTCTCTGGCTCCAGAATCCT
	4F	TAGACTCTCTACTATGGTGTCA
	4R	TAGGGCCAATGAAACAGCCAA
	5F	AGCTGTGACAAGCTACTTGGT
	5R	AATGTGCAAATCCCGAATTAACT
	6F	AGACAGGAACTGTTAATGTCCT
	6R	TGTAGGAAGATGATGCTGGGA

Primer name		Primer sequence
SDHD	1F	TGACCTTGAGCCCTCAGGAA
	1R	TCAGGGTGGGAAGACCCCT
	2F	GATCATCCTAATGACTCTTTCC
	2R	AGCAGCAGCGATGGAGAGAA
	3F	CTTTTATGAATCTGGTCCTTTTTG
	3R	CAACTATATTTGGAATTGCTATAC
	4F	TGATGTTATGATTTTTTCTTTTTCT
	4R	CAATTCTTCAAAGTATGCAGTCA
SDHAF2	1F	AGTCCGGACTAGGCCCGAA
	1R	ACGCTAGAACGTTCTCTCTCCT
	2F	TGATAGCGATGATAGTCGTCATT
	2R	GAGTCTCTCTGAACCTACTCT
	3F	AGAGTAGGTTCAGAGAGACTC
	3R	TCAAATCAGCCTAAACTGTCCTA
	4F	AGCATTGACTGACTATGGCATAA
	4R	TAGTTACCATCCACAGACTGAA
MAX	1F	GGGACTCGGGCTTGTTGTTGTCGG
	1R	CGTCGCCCCGCTAAGAGCC
	2F	CGCTCGGGACCCCTCTACCC
	2R	GGCCCCACCTCACCTTAGTGGT
	3F	CCAGGCTGTACCCCACCCACC
	3R	AGTGCTCTGCTAAGCTCTGCAACAA
	4F	AGGCCAGGTTCCCTCACTGCC
	4R	CCCAGGTGCCAAAGCCTGACC
	5F	TCCCCAAGTCTCCAGAAGGCAGT
	5R	AGGAGGATGAGACGATGGAGACAGA
TMEM127	1F	AGGCAGAGGAGGAGGAAGC
	1R	GACCTGTGCGCAGAAAAGAC
	2F	CCCCTATCCTCTGGTGTCAA
	2R	CTGGTCCCTGGCTATCTCTG
	3F	GCCGTGAAAATTTGGTTCTC
	3R	CCACCCTGTAGCAGTTCCTC
	4F	GTGCTGTCCCTCTGTCTCCT
	4R	GAGGAGCTGCAGAGTTGAGG

AGAROSE GEL ELECTROPHORESIS

2% agarose gels were made using 1x SGTB (Grisp, Oporto, Portugal) to dissolve the agarose (Lonza) after heating, and adding GelRed (Biotium, Hayward, California) to the gel, a fluorescent nucleic acid gel stain. Sample preparation requires a mixture of 6x loading buffer (stock solution: 40mL glycerol 87%, 60mL H_2O and 0,25g bromophenol blue) and DNA in a 1:2 dilution. After loading the samples in the gel, a voltage of 120V was applied to run the samples for 25 min and the gel was analysed with UV light using the ChemiDoc™ XRS System (Bio-Rad) to confirm successful amplifications.

SEQUENCING

PCR products underwent a purification treatment using 1U/µL exonuclease I (Fermentas, Thermo Fisher Scientific) and 1U/µL shrimp alkaline phosphatase (Fermentas, Thermo Fisher Scientific) at 37°C for 20 minutes, followed by heat inactivation for 15 minutes at 80°C.

Automatic sequencing was performed using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, California), Sequencing buffer (Applied Biosystems, Carlsbad, California) and the same primers used in PCR assay, in a sequence of steps: initial denaturation for 30 sec at 95°C; 35 cycles of denaturation (10 sec at 95°C), annealing (10 sec at 55°C) and extension (2 min at 60°C); and a final extension at 60°C for 10 min. The sequencing products were finally purified with Sephadex G-50 Fine (GE Healthcare), resuspended in formamide (Applied Biosystems, Carlsbad, California), and ran in automated sequencer ABI PRISM 3130 Genetic Analyser (Applied Biosystems, Carlsbad, California).

MLPA - MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

MLPA was used to search for large deletions in *SDH* genes using P226-025R MLPA probemix lot B2 1111 (MRC-Holland, Amsterdam, Netherlands).

All DNA's used in this technique had a concentration of 10 to 50ng/µL and underwent a denaturation step at 98°C for 5min. A blank sample (without DNA) was used to ensure that all solutions from MLPA kit were not contaminated. Then, in the hybridization step, 1,5µL MLPA buffer (MRC-Holland, Amsterdam, Netherlands) and 1,5µL MLPA probemix (MRC-Holland, Amsterdam, Netherlands) was added to the DNA's at 25°C. This hybridization occurred when the samples were heated at 95°C for 60sec followed by 16-20h at 60°C. The subsequent stage was ligation which consists of adding a mix composed by 3µL ligase buffer A (MRC-Holland, Amsterdam, Netherlands), 3µL ligase buffer B (MRC-Holland, Amsterdam, Netherlands), 1µL ligase 65 (MRC-Holland, Amsterdam, Netherlands) and dH_2O at 54°C for 15min and then heated to 98°C for 5min. The final step was a PCR following "one-tube protocol" given by the manufacturer's instructions that consists of preparing a mix with 4µL Salsa PCR buffer (MRC-Holland, Amsterdam, Netherlands) and dH_2O, added to the samples at 60°C. 2µL Salsa PCR primer (MRC-Holland, Amsterdam, Netherlands), 2µL Salsa enzyme Dilution Buffer (MRC-Holland, Amsterdam, Netherlands), 0,5µL Polymerase (MRC-Holland, Amsterdam, Netherlands) and dH_2O were mixed and added to each one of the PCR tubes that consequently underwent 35 cycles of denaturation (30 sec at 95°C), annealing (30sec at 60°C) and extension (60sec at 72°C) and a final extension step at 72°C for 20min.

Finally, 0,5µL size standard 500LYZ 3130 (Applied Biosystems, Carlsbad, California), dH_2O and formamide (Applied Biosystems, Carlsbad, California) were added to MLPA/PCR products and the fragments were analysed using ABI-Prism 310 Genetic Analyser (Applied Biosystems, Carlsbad, California).

MLPA results were analysed by Gene Mapper Applied Biosystems, Carlsbad, California), Peak Scanner Applied Biosystems, Carlsbad, California) and Coffalyser Softwares (MRC-Holland, Amsterdam, Netherlands).

Coffalyser software performed the ratios calculation for each probe, specific for chromosome positions comprising *SDHB, SDHC, SDHD* and *SDHAF2* exons. After performing MLPA for three times under the same conditions, mean ratios were calculated for all probes of each sample (Table 6 in the Results part). Results interpretation was based on information available by MRC-Holland which associates generated ratios with copy number status (Table 4).

Table 4 – Interpretation of ratios calculated by MLPA assay.

Copy Number Status	Ratios
Normal	0,85 – 1,15
Heterozygous Deletion	0,35 - 0,65
Homozygous Deletion	0 – 0,35
Heterozygous Duplication	1,35 – 1,55
Homozygous Duplication	1,70 – 2,20
Equivocal Copy Number	All other values

STATISTICAL ANALYSIS

Statistical significance was examined by Pearson chi-square test and T-test for genotype-phenotype correlations. In all analysis $p<0,05$ was considered statistically significant.

PART II

PATIENTS AND CASES

The second part of this study includes 12 blood samples from Portuguese PGL patients and respective clinical information, including sex, age, tumour type and local, as well as malignancy.

METHODS

DNA ISOLATION

Genomic DNA was extracted from peripheral blood, using a salt-precipitation method. First, erythrocytes were lysed by adding 45ml of 1xAKE (stock solution: 155mM of NH_4Cl, 10mM of $KHCO_3$ and 1mM pH 7,4 of EDTA) to 5ml of blood sample for 1h at 4°C. Samples were then centrifuged (2500rpm, 20min.) to remove the erythrocytes membranes and form a *pellet* of white blood cells. Next, 1% SDS (v/v) and 180µg/ml proteinase K (Fermentas, Thermo Fisher Scientific) were added to the *pellet*, in order to lyse the membranes and digest proteins, respectively; this step was performed at 55°C, overnight with shaking. Finally, proteins were precipitated after adding a pre-heated saturated solution of 1.2M NaCl, an equal volume of chloroform and shaking for 30 minutes at room temperature. After centrifugation (2500rpm, 10min.), supernatant was removed to another tube and the DNA was precipitated by adding isopropanol (1x volume); DNA *pellet* was then washed in 70% ethanol, eluted in deionized water and quantified in NanoDrop Spectrophotometer ND-1000 (ThermoScientific, Fremont, USA).

PCR / AGAROSE GEL ELECTROPHORESIS / SEQUENCING / STATISTICAL ANALYSIS

DNA amplification, gel electrophoresis and sequencing techniques, as well as statistical analysis were performed according to the same methodology explained in the previous part.

5. RESULTS

PART I

PATIENT AND TUMOUR CHARACTERISTICS

Clinical features

The mean age at diagnosis was 48 years with a range from 19 to 75 years. There was a similar prevalence of male and female patients with PCC (49% of females and 51% of males). The majority of patients had unilateral tumours (93%), followed by bilateral tumours (5%) and only one case of multiple PCC (2%); the mean tumour size was 5,03cm. From the 61 tumours, the majority were located in the right adrenal gland (56%) and the others were located in the left side (38%); in four tumours (6%) no information about the location was available. Additionally, tumours developed in the right adrenal gland (mean=4,4cm) were significantly minor than those within the left adrenal gland (mean=6,1cm) (p=0,006). Metastases were found in only three patients with PCC (5%): one had liver metastasis, another had liver, lung and bone metastasis and the third patient had metastasis at the opposite side of the adrenal tumour. Relapse occurred in three patients (5%).

Tumour histologic features

Using the PASS scoring method, a mean score of 4 was found taking into account all the tumours. This score labels tumours as potential aggressive (≥4) or benign (<4); in this study the frequency of cases presented as potential aggressive tumours was 56% and benign tumours was 44%.

The results showed that nuclear hyperchromatosis (71%), spindle cells (64%) and cellular pleomorphism (53%) were the features more frequently present in this series of PCC's. Other histologic observations were also found: 30% of the cases exhibited diffuse growth, 23% showed capsular invasion, cellular monotony was present in 20% of the tumours, high cellularity was present in 13% of the tissues, 10% of the cases had periadrenal adipose tissue infiltration, vascular invasion was present in 8%, cellular necrosis and mitotic figures (>3/10HPF) were in 3% of the cases and finally atypical mitotic figures were not seen in any tumour. More than 3 mitotic figures/10HPF were present only in two tumours with 7-8 and 4 mitotic figures, each; cellular necrosis was found in only two cases.

Tumour microvessel density

Under the capsule, CD31 showed a mean vessel density of 40,34 vessels/mm^2, while CD105 showed a mean vessel density of 23,56 vessels/mm^2; in the middle of the tumour CD31 marked a mean vessel density of 46,09 and CD105 presented a mean vessel density of 33,09.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry of 61 paraffin-embedded tissues from 57 PCC patients was performed for SDHB and scored in positive (1 plus, 2 plus or 3 plus) or negative according to the amount of antibody-antigen binding for SDHB protein in tumour cells (Table 5). This procedure was performed in order to identify the cases with germline *SDH* mutations, since the presence of such mutations are closely associated with loss of SDHB expression. The staining revealed 3 negative tumours (5%) (Figure 4) and 58 positive cases (95%). Positive cases showed cytoplasmic and granular staining of SDHB, consistent with a mitochondrial pattern. Among the 58 positive cases, 10 were scored as 1 plus (17%) (Figure 6A), 13 with 2 plus (23%) (Figure 6B) and the majority of the cases with a 3 plus score (57%) (Figure 6C). Only 2 cases were classified as 2 and 3 plus simultaneously, since it was difficult to differentiate them in one of the classes (3%). The three cases without SDHB expression did not show evidence of MEN2, NF1 or VHL.

Table 5 – Immunohistochemistry results for SDHB expression in 61 tumour tissues from 57 PCC patients.

SDHB expression	All tumours (n=61)
Negative	3 (5%)
Positive	58 (95%)
1+	10 (17%)
2+	13 (23%)
2+/3+	2 (3%)
3+	33 (57%)

Immunohistochemistry for SDHA protein was performed in the three SDHB negative cases, in order to discard *SDHA* as the mutated gene causing PCC. Whenever there is a mutation in *SDHB, SDHC* or *SDHD*, there is loss of SDHB expression, while SDHA is still expressed; on the other hand, in the presence of a *SDHA* mutation there is loss of both SDHB and SDHA. The immunohistochemistry showed positive expression of SDHA protein in the three SDHB-negative cases, discarding any *SDHA* mutations (Figure 5).

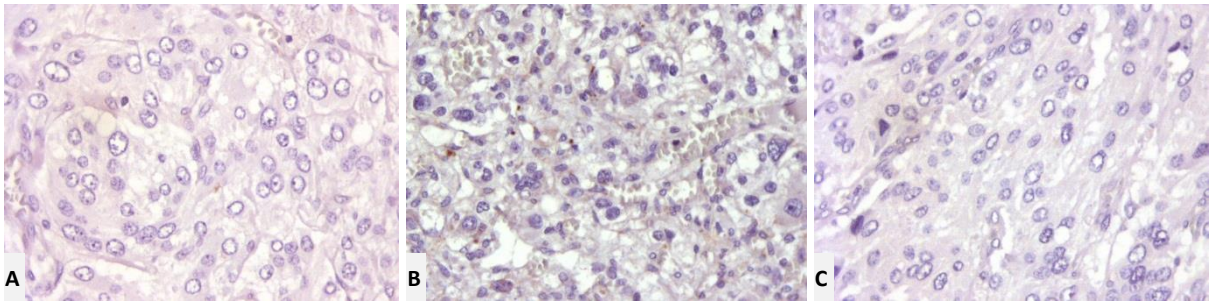


Figure 4 - Negative immunohistochemistry for SDHB protein in three PCC cases.

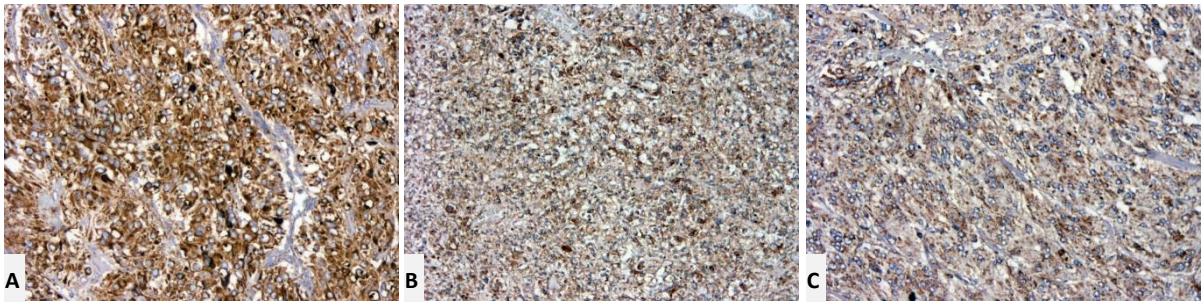


Figure 5 – Positive immunohistochemistry for SDHA protein in the three PCC cases without SDHB expression.

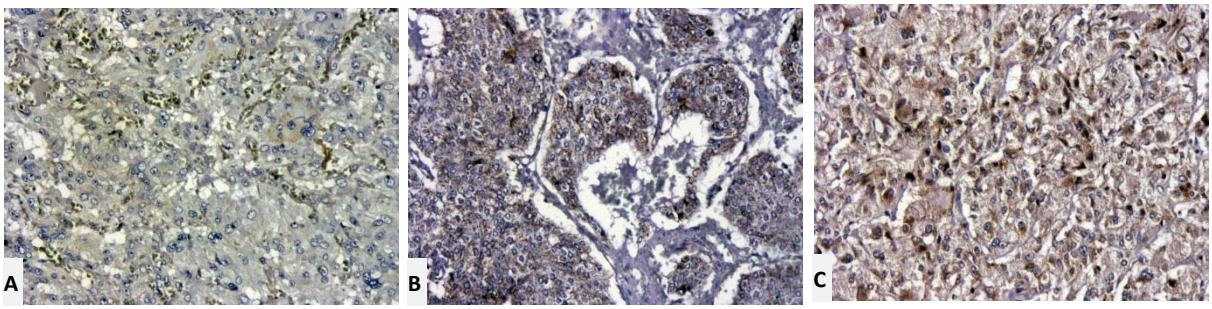


Figure 6 – Positive immunohistochemistry for SDHB protein in three PCC cases. A – 1 plus score; B – 2 plus score; C – 3 plus score

MOLECULAR GENETICS OF PCC CASES

Cases with positive familial history

Regarding the molecular genetics approach, the first criterion used to select the most probable gene was the presence of familial/personal history compatible with NF1, MEN2 or VHL.

Four NF1 patients (one of them with a bilateral PCC) were diagnosed solely based on clinical criteria, since *NF1* gene is very large and without hot spots, making it extremely difficult to perform genetic screening of the disease. Two cases (both with bilateral PCC's) were clinically diagnosed with MEN2 syndrome and both were found to carry a germline *RET* mutation: one patient carried a missense mutation in exon 16 - c.2753T>C, p.M918T - indicating a MEN2B situation (Figure 7), while the other patient harboured a missense mutation in exon 11 – c.1901G>A, p.C634Y - that is indicative of MEN2A syndrome (Figure 8). The patient with VHL syndrome was screened for mutations in *VHL* gene, but PCR amplification of tumour DNA was not possible, perhaps due to poor quality of material.

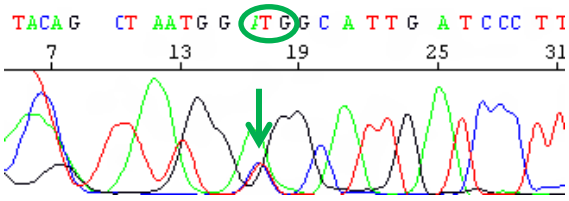


Figure 7 – *RET* mutation in exon 16 (c.2753T>C, p.M918T).

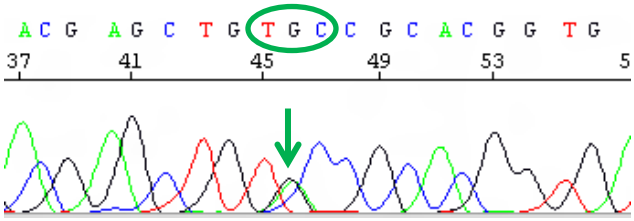


Figure 8 - *RET* mutation in exon 11 (c.1901G>A, p.C634Y).

Cases without familial history (apparently sporadic)

In the cases that did not show evidence of NF1, MEN2 or VHL, the absence of SDHB expression was used to select the cases to be screened for *SDH* mutations. The three PCC cases that showed loss of protein expression were tested for mutations in *SDH* genes, in order to find the underlying pathogenic mutation variant.

The first genetic alteration to be assessed was a large deletion in the *SDHB* gene, comprising the promoter region and exon 1, removing 15678bp; this alteration was recently identified in a large proportion of Portuguese PGL/PCC patients and previously described by Cascón et al. All three PCC cases were negative for this deletion. Next, the entire coding regions and exon-intron boundaries of *SDHB, SDHD, SDHC* and *SDHAF2* genes were sequenced, but no variants were detected. The next step was to look for the presence of large deletions using MLPA assay, which includes a probemix for all *SDH* genes.

Two out of three samples were identified as carrying large deletions: one of them had an exon 4B deletion in *SDHC* and an exon 4 deletion in *SDHD*; the second one had an exon 4B deletion in *SDHC* (Table 6). In the remaining sample, despite the absence of SDHB expression, no *SDH* mutations were disclosed (Table 6).

SDHC exon 4B deletion comprised 400 nucleotides deletion and was detected in two samples, one of them also carrying the large deletion mentioned above. This SALSA MLPA probe in the chromosome position 01q23.3, includes 2 ligation sites to cover a large area (>10kb) that comprise introns surrounding the exon 4. Also, the probe has a quite low Tm and thus, can be variable, as target sequence can carry a SNP which easily affects the binding of the probe, resulting in a reduced probe signal. To obtain specific conclusions for this deletion, it was recommended to check the target sequence for SNP's, which was not possible to perform. Thus, this deletion was not considered as a true pathogenic variant for this study.

Although only one sample was considered as carrying an *SDH* mutation, the three cases with negative SDHB immunohistochemistry were considered for genotype-phenotype correlations, as they had absent SDH expression.

Table 6 – MLPA results for *SDH* large deletions.

Exon	Chromosome position	Length (nt)	Ratios		
			Sample 1	Sample 2	Sample 3
***SDHB* Exon 1A**	01p36.13	344	1,79	1,69	2,31
***SDHB* Exon 1B**	01p36.13	166	1,61	1,19	1,85
***SDHB* Exon 2**	01p36.13	226	1,37	1,18	1,15
***SDHB* Exon 3**	01p36.13	208	1,06	0,93	0,79
***SDHB* Exon 4**	01p36.13	263/373	1,23	0,87	0,91
***SDHB* Exon 5**	01p36.13	256	1,00	1,21	0,79
***SDHB* Exon 6**	01p36.13	238	0,98	1,08	0,88
***SDHB* Exon 7**	01p36.13	328/337	1,15	1,19	1,04
***SDHB* Exon 8**	01p36.13	190	1,17	1,05	0,84
***SDHC* Promotor**	01q23.3	138/142	1,11	1,18	2,30
***SDHC* Exon 1**	01q23.3	355	1,28	1,02	2,07
***SDHC* Exon 2**	01q23.3	182/184	0,80	0,79	0,73
***SDHC* Exon 3A**	01q23.3	382	0,94	0,71	0,81
***SDHC* Exon 3B**	01q23.3	270	0,90	0,70	0,80
***SDHC* Exon 4A**	01q23.3	148	1,05	0,85	0,85
***SDHC* Exon 4B**	01q23.3	400	1,19	0,25	0,30
***SDHC* Exon 5**	01q23.3	292	0,93	0,81	0,92
***SDHC* Exon 6**	01q23.3	247	0,81	1,01	1,23
***SDHD* Promotor**	11q23.1	318/326	0,90	0,68	1,69
***SDHD* Exon 1**	11q23.1	160/178	1,00	0,73	1,98
***SDHD* Exon 2**	11q23.1	172	0,97	0,84	1,41
***SDHD* Exon 3**	11q23.1	311/319	0,81	0,66	1,00
***SDHD* Exon 4**	11q23.1	214	0,84	0,06	0,84
***SDHAF2* Exon 1**	11q12.2	160	0,91	0,90	2,03
***SDHAF2* Exon 3**	11q12.2	391	0,83	0,71	1,19
***SDHAF2* Exon 4**	11q12.2	418	0,87	0,70	1,55
SDHAF1	19q13.12	136	1,2	1,07	1,13

Finally, the 48 remaining cases (61 tumour tissues excluding the three SDH, five NF1, four MEN2 and one VHL) were submitted to Sanger sequencing for *MAX* and *TMEM127* genes, as they constitute two novel susceptibility genes related with PCC/PGL's.

For the *MAX* gene, 26 tumour DNAs were sequenced for all 5 exons; for technical reasons, in 15 tumours only part of the gene was sequenced, while in seven cases *MAX* sequencing could not be performed. For the *TMEM127* gene, all exons, except for exon 1 (non-coding exon), were sequenced

in 14 tumour DNAs; for technical reasons, in 28 cases *TMEM127* was incompletely sequenced and in six cases were impossible to perform sequencing.

No pathogenic mutation was detected in any of the two genes. A synonymous variant in exon 4 of *TMEM127* gene (c.621G>A; p.A207A) was detected in 7 cases (12%) (Figure 9); this alteration is a previously reported SNP, with an allele frequency of 18% in European populations (Neumann et al. 2011).

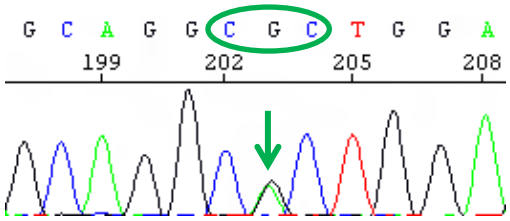


Figure 9 – SNP in *TMEM127* exon 4 (c.621G>A; p.A207A).

Genotype-phenotype correlation

After genetic analysis, genotype was associated with phenotype by comparing mutated with non-mutated cases (Table 7, 8 and 9). Mutated cases comprised 10 patients (18%) with familial history: three of them with loss of SDH expression, four NF1 patients, two MEN2 with missense mutations in *RET* gene and one VHL with possible loss of gene expression. The remaining 47 (82%) cases comprised non-mutated cases and thus termed sporadic cases.

As depicted in Table 7, the mean age at diagnosis was not significantly different between mutated and non-mutated cases, although mutated cases had a lower mean age at diagnosis than non-mutated cases (41 vs. 50, respectively, p=0,090). Among the mutated cases, SDH patients had a mean age at diagnosis of 38 years, MEN2 patients presented a mean age of 29, NF1 patients showed a mean age of 54 and the VHL case developed in a 19 years old individual. Excluding NF1 patients that had an age at diagnosis comparable to that of sporadic cases, mutated cases had a significantly younger age at diagnosis than cases without familial history (p=0,006).

Among the non-mutated cases there was a similar prevalence of male and female patients with PCC (51% of females and 49% of males); among the mutated cases there was higher frequency of males than females (40% of females and 60% of males), but without statistical significance (p=0,730). MEN2 patients had an equal frequency of males and females (50%), NF1 cases comprised more males (75%) than females (25%) and *VHL* and *SDH* mutated cases affected only male patients.

There was a significantly higher frequency of bilateral tumours in mutated cases, when compared to non-mutated cases (30% vs. 0%, p=0,015). The only multiple tumour was found in a non-mutated case. SDH patients had only unilateral tumours, MEN2 cases presented only bilateral PCC's, NF1 patients had 3 unilateral (75%) and 1 bilateral tumour (25%) and the VHL patient had an unilateral PCC.

The mean tumour size of mutated cases was 4,42cm and non-mutated cases had a mean tumour size of 5,20cm, without statistically significant differences (p=0,295). The larger tumours belonged to NF1 patients with a mean size of 5,26cm, followed by MEN2 tumours with a mean size of 4,88cm, SDH tumours with 3,27cm and finally the only VHL tumour with a size of 1,8cm.

Mutated and non-mutated cases followed the same tendency, with a predominance of tumours in the right side and thus without significant differences for tumour location (p=0,657). MEN2 cases had equal frequencies for the two adrenal glands since the patients had bilateral tumours, NF1 patients had more frequency of tumours in the right (60%) than in the left adrenal gland (40%) and SDH and VHL cases had tumours located only in the right adrenal gland.

Metastasis were found in only three patients with PCC (6%) all with sporadic disease; relapse occurred in three patients, one of them a NF1 case (10%) and the other two sporadic cases (4%).

Table 7 – Correlation of clinical features between mutated and non-mutated patients.

Clinical features	Non-mutated patients (n=47)	Mutated cases					P-value
		All patients (n=10)	MEN2 patients (n=2)	NF1 patients (n=4)	VHL patient (n=1)	SDH patients (n=3)	Mutated patients Vs Non-mutated patients
Gender							
F	24 (51%)	4 (40%)	1 (50%)	3 (75%)	0	0	p=0,730
M	23 (49%)	6 (60%)	1 (50%)	1 (25%)	1 (100%)	3 (100%)	
Age							
Range	19-75	20-70	20-37	45-70	-	28-50	p=0,090
Mean	50	41	29	54	19	38	
Tumour Type							
Unilateral	46 (98%)	7 (70%)	0	3 (75%)	1 (100%)	3 (100%)	**p=0,015**
Bilateral	0	3 (30%)	2 (100%)	1 (25%)	0	0	
Multiple	1 (2%)	0	0	0	0	0	
Total number of tumours	48	13	4	5	1	3	
Tumour Size							
Range	1,5-13,0	1,5-10,0	1,5-8,0	2,8-10,0	-	2,8-3,5	p=0,295
Mean	5,20	4,42	4,88	5,26	1,8	3,27	
Tumour Location							
Left	19 (40%)	4 (31%)	2 (50%)	2 (40%)	0	0	p=0,657
Right	25 (52%)	9 (69%)	2 (50%)	3 (60%)	1 (100%)	3 (100%)	
No info	4 (8%)	0	0	0	0	0	
Metastasis	3 (6%)	0	0	0	0	0	
Relapse	2 (4%)	1 (10%)	0	1 (25%)	0	0	

Table 8 shows that both mutated and non-mutated cases had a mean PASS score of 4. Tumours from mutated cases did not show high cellularity, vascular invasion and periadrenal adipose tissue infiltration. However, diffuse growth, cellular monotony, presence of spindle cells and pleomorphism were more frequent comparing with non-mutated tumours. More than 3 mitotic figures/10HPF were present only in two tumours, one sporadic and one familial (MEN2) with 7-8 and 4 mitotic figures, respectively; cellular necrosis was found in only two cases belonging to a non-mutated tumour and the same MEN2 case previously referred. The other features had higher frequencies in mutated tumours, namely capsular invasion and hyperchromatosis. However, no significant differences were seen in any histologic feature comparing mutated with non-mutated cases.

Table 8 - Correlation of tumour histologic features between mutated and non-mutated tumours.

Tumour histologic features	Mutated tumours (n=13)[a]	Non-mutated tumours (n=48)[b]	P-value Mutated tumours Vs Non-mutated tumours
Diffuse growth	4 (31%)	14 (30%)	n.s.s.
Single cell necrosis	1 (8%)	1 (2%)	p=0,384
High cellularity	0	8 (17%)	p=0,184
Cellular monotony	3 (23%)	9 (19%)	p=0,707
Tumour spindle cells	11 (85%)	28 (58%)	p=0,109
More than 3 mitotic figures/10HPF	1 (8%)	1 (2%)	p=0,384
Atypical mitotic figures	0	0	-
Periadrenal adipose tissue infiltration	0	5 (10%)	p=0,575
Vascular Invasion	0	5 (10%)	p=0,575
Capsular Invasion	2 (15%)	12 (25%)	p=0,713
Cellular pleomorphism	8 (62%)	24 (50%)	p=0,541
Nuclear hyperchromatosis	8 (62%)	35 (73%)	p=0,499
Mean PASS score (0-20)	4	4	p=0,826
PASS (<4) **Mean**	6 (46%) 3	21 (44%) 2	
PASS (≥4) **Mean**	7 (54%) 6	27 (56%) 6	

n.s.s. – not statistically significant
[a]13 tumours from 10 familial patients
[b]48 tumours from 47 sporadic patients

Finally, microvessel density was also used to compare mutated with non-mutated tumours (Table 9). Considering CD31 staining, it was shown that non-mutated cases presented significantly higher vessel density than mutated cases, under the capsule (p=0,028). The same was not observed for the CD105 marker (Table 9).

Table 9 - Correlation of microvessel density between mutated and non-mutated tumours.

Number of vessels	Mutated tumours (n=13)[a]	Non-mutated tumours (n=48)[b]	P-value
			Mutated tumours Vs Non-mutated tumours
Chalkley score/mm^2			
Under the capsule			
CD31 Mean	33,03	42,32	**p=0,028**
CD105 Mean	20,93	24,27	p=0,410
Middle of the tumour			
CD31 Mean	39,90	47,76	p=0,083
CD105 Mean	32,12	33,36	p=0,798

[a]13 tumours from 10 familial patients
[b]48 tumours from 47 sporadic patients

PART II

PATIENT AND TUMOUR CHARACTERISTICS

For the second part of this Thesis, 12 blood samples from Portuguese PGL/PCC patients were screened for *SDH* genes.

Clinical information including sex, age, tumour type and localization is summarized in Table 10.

This series was constituted of PGL's and PCC's. Three PGL's were located in the head and neck region (50%), one in the pancreas (17%) and one in the retroperitoneum (17%), while one case did not have information regarding location; the remaining six cases were PCC's. There was an equal female to male ration and the mean age at diagnosis was 41 years with a range between 15 and 63. There were two malignant tumours (17%), both PCC cases, one male patient with 15 years old and a female patient with 44 years old.

Two cases had familial history, one bilateral PCC belonging to a male patient with 28 years old whose index case was his father presenting also a bilateral PCC; the other familial case was a PGL from a male patient with 15 years old whose index case was his mother.

Table 10 – Clinical features from 12 PGL/PCC patients.

Clinical features	All patients (n=12)
Gender	
F	6 (50%)
M	6 (50%)
Age	
Range	15-63
Mean	41
Tumour Type	
PGL	6 (50%)
Head and Neck	3 (50%)
Pancreas	1 (17%)
Retroperitoneal	1 (17%)
Extra-adrenal	1 (17%)
PCC	6 (50%)
Bilateral	1 (17%)
Malignancy	2 (17%)

MOLECULAR GENETICS

In accordance with Part I of the Results, the first molecular alteration to be assessed was the *SDHB* large deletion comprising promoter region and exon 1, as it is a frequent variant in northern Portuguese PGL/PCC tumours. Afterwards, in cases that did not present this large deletion, *SDHB*, *SDHD* and *SDHC* genes were tested for mutations, in this order.

The results showed that two cases (17%) carried the *SDHB* large deletion (c.1-10413_73-3866del), one being a bilateral head and neck PGL from a 53 year old female patient (MASC) and the other one a patient with malignant PCC with 15 years (MAMR). As the deletion encompasses a large region of *SDHB* gene, primers were designed selectively to amplify the deleted allele, only when the deletion is present. Thus, when amplification occurred, deletion was present and when deletion was absent, primers were unable to amplify the large region (Figure 10). Sequencing the region amplified by this pair of primers showed the breakpoint of the deletion (Figures 11 and 12).

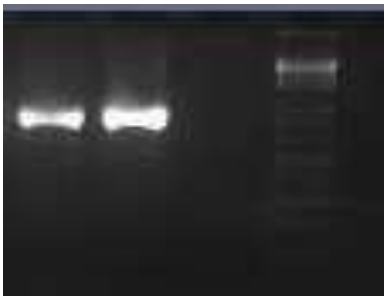


Figure 10 – Gel electrophoresis for *SDHB* exon 1 and promoter deletion (sample, control, blank and ladder).

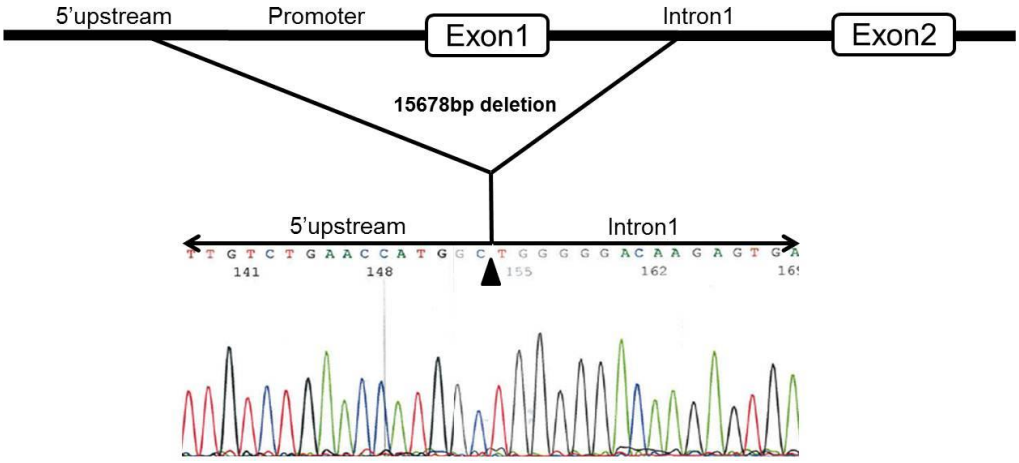


Figure 11 – Scheme of *SDHB* large deletion (c.1-10413_73-3866del).

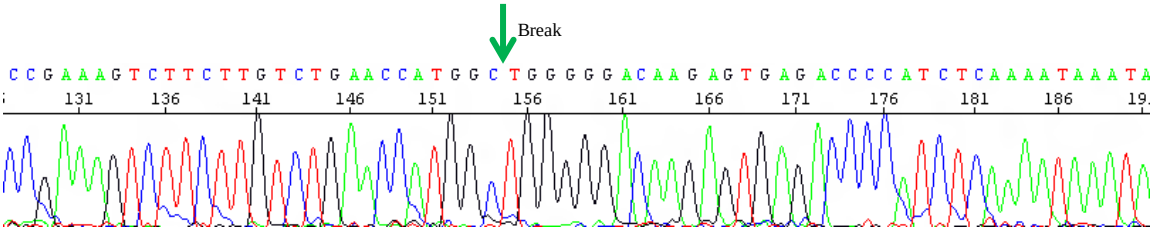


Figure 12 – Breakpoint in *SDHB* exon 1 + promoter large deletion of MASC and MAMR patients.

Additionally , one (8%) patient with head and neck PGL (APFC) was found to harbour a missense mutation in exon 1 of *SDHB* gene (c.32G>A; p. R11H) (Figure 13); another patient (8%) with a retroperitoneal PGL (PMOV), presented a frameshift mutation in exon 6 of *SDHB* gene (c.591 del.C; p.S198AfsX22) (Figure 14); finally, a patient (8%) with 15 years who developed PGL and had familial history (AEMS), showed a missense mutation in exon 1 of *SDHD* gene (c.3 G>C; p.M1I) (Figure 15).

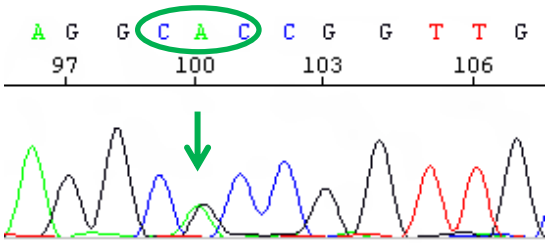


Figure 13 – Missense mutation in *SDHB* exon 1 (c.32G>A; p. R11H) in APFC patient.

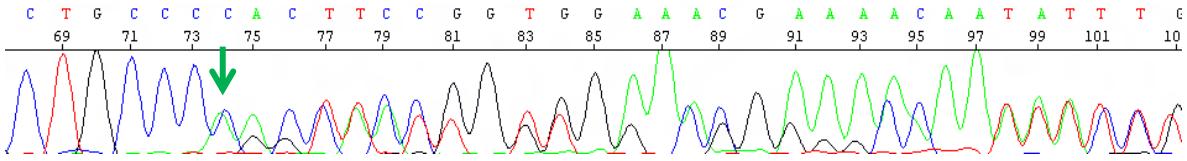


Figure 14 – Frameshift mutation in *SDHB* exon 6 (c.591 del.C; p.S198AfsX22) in PMOV patient.

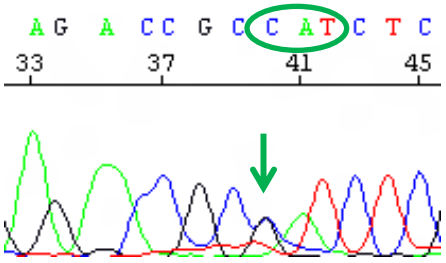


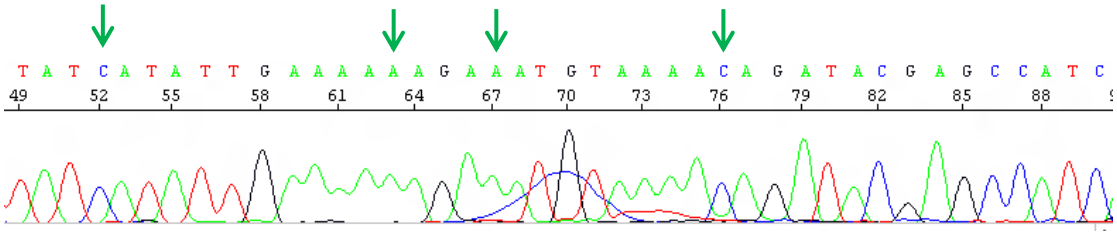
Figure 15 – Missense mutation in *SDHD* exon 1 (c.3 G>C; p.M1I) in AEMS patient. (reverse sequence)

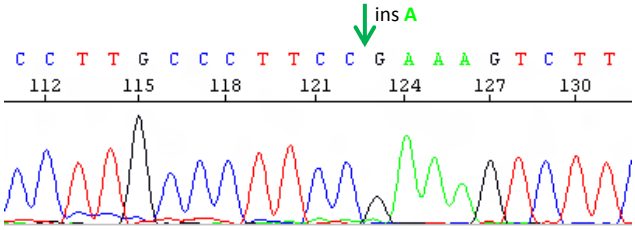
Since previous studies from our group had found a high prevalence of the large *SDHB* deletion, haplotype analysis was performed to address whether this deletion had a founder effect in Portuguese patients. For that matter, the up- and downstream flanking regions of the deleted allele were sequenced and the status of already annotated SNP's was determined (Figure 16). It was observed that the deleted allele had no haplotypic variation, comparing with control population that showed six different haplotypes (Table 11).

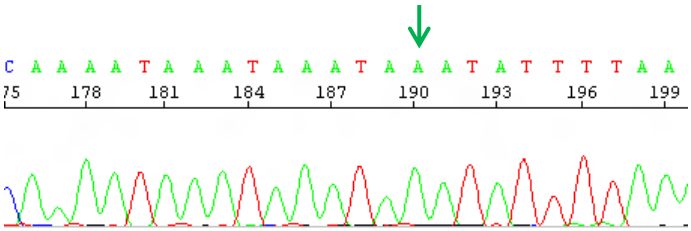
Table 11 – Haplotypes detected in the control population.

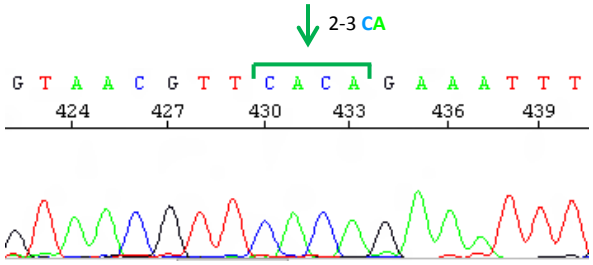
Haplotypes	SNP's							Frequency (%)
	rs1569754	rs3946080	rs2143811	rs5772743	rs7545518	rs7545499	rs7536679	n=100 chr
Hap1	C	A	C	A	A	A	T	4.0
Hap2	C	A	C	A	G	G	C	23.0
Hap3*	C	A	C	-	A	A	T	47.0
Hap4	C	A	C	-	G	G	C	12.0
Hap5	T	G	T	A	A	A	T	1.0
Hap6	T	G	T	A	G	G	C	13.0

* haplotype 3 is the one present in the deleted alleles









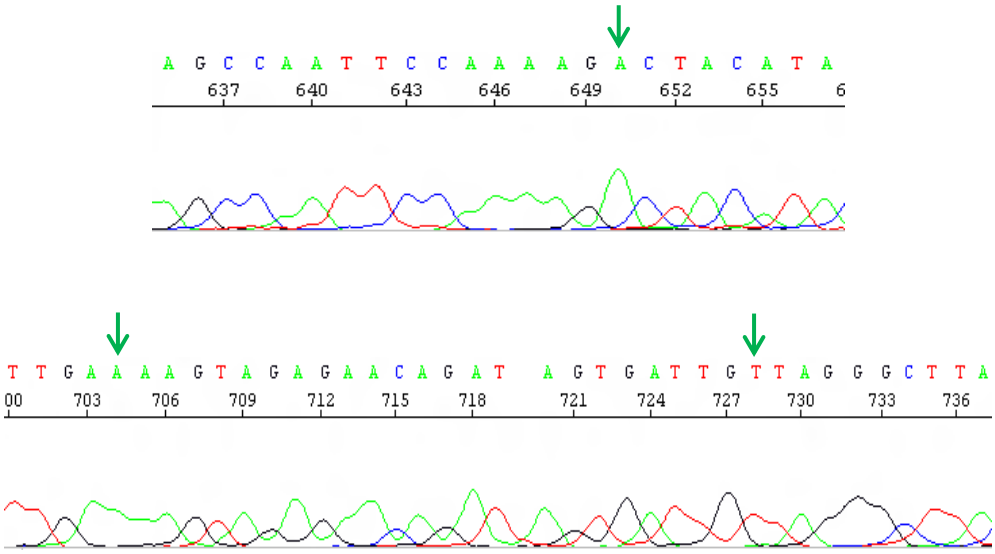


Figure 16 – Location of the SNP (arrows) used to define the haplotype of the *SDHB* large deletion comprising exon 1 and promoter.

Furthermore, we decided to analyse PMFS patient for mutations in *MAX* gene as it had familial history, young age and a bilateral PCC. A nonsense mutation in *MAX* exon 3 was detected (c.97C>T; p.R33X), resulting in a premature stop codon and a truncated protein (Figure 17).

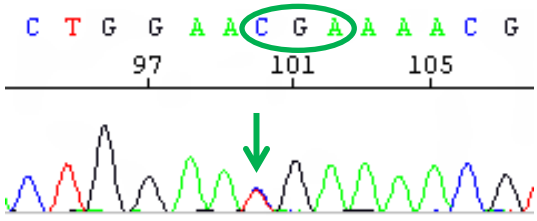


Figure 17 – Nonsense mutation in *MAX* exon 3 (c.97C>T; p.R33X) from PMFS patient.

In this series of 12 PGL/PCC's, five different pathogenic variants were uncovered in six patients (50%), four of them point mutations (80%) - two missense mutations (50%), one nonsense mutation (25%) and one frameshift mutation (25%) -, and one large deletion (20%) present in two patients.

Three out of five mutations occurred in the *SDHB* gene (60%), one in *SDHD* gene (20%) and another one in *MAX* gene (20%).

Two non-pathogenic variants were also identified, both in *SDHB* gene: the first one in exon 1 (c.18 C>A; p.A6A) occurring in two patients, one with head and neck PGL (APFC) and carrying also a missense mutation (c.32 G>A; p.R11H) and the other one with PCC (PJSA) (Figure 18); the second variant was located in exon 5 (c.487 T>C; p.S163P) and showed up in one patient with pancreatic PGL (BMSO) (Figure 19).

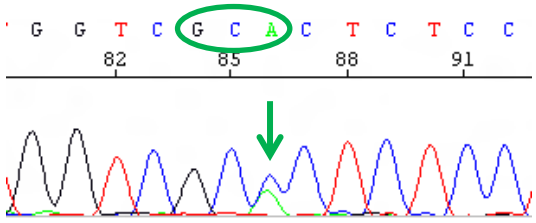


Figure 18 – SNP in *SDHB* exon 1 (c.18 C>A; p.A6A) from APFC and PJSA patients.

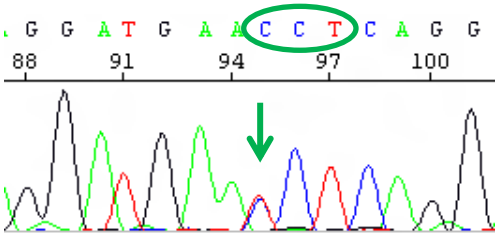


Figure 19 - SNP in *SDHB* exon 5 (c.487 T>C; p.S163P) from BMSO patient.

Table 12 summarizes all genetic variants carried by this series of 12 PCC/PGL's. So, seven different genetic variants were found in this series of PGL/PCC patients, five of them pathogenic mutations (71%) and two non-pathogenic point mutations (29%). In total, eight patients (67%) carried at least one genetic variation, five of them as PGL cases (63%) and three PCC's (37%).

Table 12 – Genetic variants carried by PGL/PCC patients.

Clinical features					*SDH* mutations						*MAX* mutations		
					SDHB			*SDHD*					
I.D.	Sex	Age	Tumour	Additional features	Exon	Nucleotide change	Protein change	Exon	Nucleotide change	Protein change	Exon	Nucleotide change	Protein change
HMO	F	44	PCC	Malignant	-	-	-	-	-	-	-	-	-
APFC	M	35	HN PGL	-	1	c.18 C>A c.32 G>A	p.A6A p. R11H	-	-	-	-	-	-
PMFS	M	28	Bilateral PCC	Index case – father	-	-	-	-	-	-	3	c.97C>T	p.R33X
AEMS	M	15	PGL	Index case – mother	-	-	-	1	c.3 G>C	p.M1I	-	-	-
MASC	F	53	HN PGL	-	1 + promoter	c.1-10413_73-3866del	Exon 1 deletion	-	-	-	-	-	-
MAMR	M	15	PCC	Malignant	1 + promoter	c.1-10413_73-3866del	Exon 1 deletion	-	-	-	-	-	-
IMPS	F	42	PCC	-	-	-	-	-	-	-	-	-	-
MJCBS	F	52	PCC	-	-	-	-	-	-	-	-	-	-
MRPT	F	57	HN PGL	-	-	-	-	-	-	-	-	-	-
BMSO	F	56	Pancreas PGL	-	5	c.487 T>C	p.S163P	-	-	-	-	-	-
PMOV	M	31	Retroperitoneal PGL	-	6	c.591 del.C	p.S198AfsX22	-	-		-	-	-
PJSA	M	63	PCC	-	1	c.18 C>A	p.A6A	-	-	-	-	-	-

Genotype-phenotype correlation

In order to correlate the genotype with the phenotypic presentation of the disease, the clinical features of patients with germline mutations were compared with those of patients without germline mutations (Table 13). Mutated patients were also divided according to the mutated gene; only *SDHB* mutated patients were compared with non-mutated cases as *SDHD* and *MAX* positive cases comprised only one patient.

Non-mutated cases were more frequently female (83%) than male patients (17%), had a mean age at diagnosis of 52 years and more frequently developed PCC's (67%) than PGL's (33%). On the other hand, mutated cases had an inversion in sex ration and tumour location compared with non-mutated cases. They comprised more male (83%) than female patients (17%) and more PGL's (67 %) than PCC cases (33%); mean age at diagnosis was significantly (p=0.006) lower in mutated cases than in non-mutated cases (30 vs. 52 years, respectively).

Comparing *SDHB* mutated cases with non-mutated ones, more male (75%) than female patients (25%) were affected, with a mean age of 34 years old and comprising more PGL's (75%) than PCC tumours (25%). Significant differences appeared for mean age at diagnosis, as *SDHB* mutated cases had younger ages than non-mutated cases (p=0,035).

There were two malignant tumours in this series, one in a patient without germline mutations and another in a patient with a *SDHB* large deletion (c.1-10413_73-3866del).

Table 13 – Correlation between non-mutated patients with all mutated and *SDHB* mutated patients.

Clinical features	Non-mutated patients (n=6)	Mutated patients				P-value	
		All mutated patients (n=6)	*SDHB* mutated patients (n=4)	*SDHD* mutated patient (n=1)	*MAX* mutated patient (n=1)	Non-mutated Vs Mutated patients	Non-mutated Vs *SDHB* mutated patients
Gender							
F	5 (83%)	1 (17%)	1 (25%)	0		p=0,080	p=0,190
M	1 (17%)	5 (83%)	3 (75%)	1 (100%)	1 (100%)		
Mean Age	52	30	34	15	28	**p=0,006**	**p=0,035**
Tumour Location							
PGL	2 (33%)	4 (67%)	3 (75%)	1 (100%)	0		
Head and Neck	1 (50%)	2 (50%)	2 (67%)	0	0		
Pancreas	1 (50%)	0	0	0	0		
Retroperitoneal	0	1 (25%)	1 (33%)	0	0	p=0,567	p=0,542
Extra-adrenal	0	1 (25%)	0	1 (100%)	0		
PCC	4 (67%)	2 (33%)	1 (25%)	0	1 (100%)		
Bilateral	0	1 (100%)	0	0	1 (100%)		
Malignancy	1 (14%)	1 (20%)	1 (25%)	0	0		

Polymorphic variants were excluded for this analysis

6. DISCUSSION

This Thesis aimed to progress in the understanding of the molecular genetics of PCC and PGL, taking into account that PCC and PGL are the human neoplasias with the largest number of susceptibility genes. We have studied two different populations, one from Poland – composed of PCC - and another from Portugal – composed of PCC and PGL.

The first aim was to identify loss of SDH enzyme expression in the tumour tissue from the Polish PCC series. After discovering three cases with loss of SDH expression, these were picked for *SDH* mutation screening, in order to explain the absence of protein expression. Indeed, two cases with absent SDH expression carried large deletions in *SDH* genes, specifically *SDHD* exon 4 and *SDHC* exon 4B. However, only *SDHD* exon 4 deletion was considered a mutation, since *SDHC* exon 4B probe is extremely variable and can easily be affected by a SNP carried by the target sequence (that we did not assess). Besides, one of the samples with negative SDHB immunostaining carried both the *SDHD* exon 4 deletion (which was confirmed three times, with different probesets of the MLPA reaction) and the *SDHC* exon 4B deletion (that only appeared once), making the *SDHC* 4B deletion a probable artefact. Thus, this exon 4B deletion in *SDHC* was not considered as a true genetic variant.

The deletion encompassing *SDHD* exon 4, results in the removal of the terminal coding exon which might explain the absence of SDH expression. Mitochondrial complex II has two cytochrome b subunits composed of large (cybL – SDHC) and small (cybS – SDHD) subunits that act as hydrophobic membrane anchor peptides and are essential for the interaction between the complex and quinone species. Each one has three transmembrane helices (I, II, III, and IV, V, VI): helices II and V provide a histidine ligand to the heme closest to the [3Fe-4S] cluster within SDHB; and helices I and IV provide the histidines for lower potential heme located towards the membrane surface (Hirawake et al. 1999; Ackrell 2000). SDHC and SDHD proteins also contain two ubiquinone-binding sites (Horsefield et al. 2006; Welander et al. 2011; Fishbein et al. 2012).

Thus, the removal of the terminal coding exon from *SDHD* gene will possibly result in alterations of protein transmembrane helices, consequently changing the attachment of SDHB and SDHA to the inner membrane and modifying the electrons transfer from iron-sulphur clusters within SDHB. As ubiquinone-binding sites within SDHC and SDHD can also be affected, the final acceptor ubiquinone will possibly be unable to accept electrons transferred from flavin by iron-sulphur clusters. Thus,

electron transport chain will be deficient or blocked, resulting in absent generation of an electrochemical proton gradient across the inner membrane of mitochondria, which is essential for ATP synthesis in the oxidative phosphorylation (Lodish et al. 2000). As complex II couples the oxidation of succinate to fumarate with the reduction of quinone to quinol, in the Krebs cycle and in the electron transport chain, respectively, the referred changing events might also abolish association between these two metabolic processes.

A large deletion in *SDHD* exon 4 was reported by Janecke et al. in two familial PGL's from Austria with a founder effect. This was the first large *SDHD* deletion characterized at the sequence level. This gross deletion removes the terminal coding exon within a region of high Alu content. Human genome has only 10% of Alu repeats and they constitute 24% of the *SDHD* genomic sequence, between the gene transcription start site and the end of terminal coding exon; 42% of the genomic sequence is flanking the terminal exon of *SDHD*, where the breakpoint of the deletion occurred. As Alu-mediated recombination events are responsible for large germline deletions, high Alu content in the terminal coding exon of *SDHD* might be implicated in the pathogenesis of the deletion (Janecke et al. 2010).

Large germline founder deletions in *SDHB* and *SDHC* were found in Spain and United States (Baysal et al. 2004; Cascón et al. 2008; Ricketts et al. 2010); in *SDHD* gene, so far, only single nucleotide changes or small deletions and insertions were described as founder mutations in *SDHD* gene, first in the Netherlands and more recently in the United States, Italy, Spain and China (Taschner et al. 2001; Baysal et al. 2002; Lee et al. 2003; Simi et al. 2005; Velasco et al. 2005). The large deletion in *SDHD* exon 4 identified in a PCC Polish patient could be the same deletion reported by Janecke et al. and perhaps with a founder effect extensible from Austrian to Polish population; however further studies are needed to confirm the deletion breakpoint and variation in the flanking regions of the deleted allele.

Only one sample was found to have a *SDH* mutation, after screening for point mutations, as well as small and large deletions in all *SDH* genes. The only exception was *SDHA* that was not analysed since its expression was retained in the tumour tissue, discarding possible mutations. We are therefore missing the alternative events that are on the basis of loss of SDH expression in two PCC,

which may include deleterious mutations in untranslated, intronic, or promoter regions of the genes, or epigenetic silencing of *SDH* genes.

In addition to the three cases with SDH loss, seven cases had familial history compatible with syndromic disease: four patients were diagnosed with NF1 based on clinical criteria, two cases had clinical feature related with MEN2 syndrome and were further diagnosed by *RET* genetic testing, and the case with VHL syndrome, was screened for mutations in *VHL* gene.

Patients with MEN2 carried missense mutations in *RET* gene: one located in exon 11 (c.1901G>A, p.C634Y) and the other one in exon 16 (c.2753T>C, p.M918T). These two mutations were already reported and constitute the most common pathogenic genetic variants underlying MEN2A and MEN2B syndromes, respectively (Carlson et al. 1994; Mulligan et al. 1994).

In the VHL patient, PCR amplification of tumour DNA for *VHL* gene was not possible, which may suggest a complete loss of *VHL* gene in the tumour tissue. According to the Knudson two-hit model, biallelic *VHL* inactivation due to mutation or hypermethylation is a common event in hemangioblastomas and clear-cell renal carcinomas. Normally, in sporadic tumours both the first and second “hit” occur somatically rather than in the germline. However, in apparently sporadic PCC’s, germline *VHL* mutations are not as rare as in clear-cell renal carcinoma or hemangioblastoma and somatic *VHL* mutations are rare in truly sporadic PCC’s. It is unknown why some germline *VHL* mutations lead to PCC and yet somatic *VHL* mutations are rare. Although the reasons are still unclear, this might suggest that *VHL* inactivation during a critical developmental window or *VHL* haploinsufficiency results in PCC formation in the setting of VHL disease (Kim et al. 2004; Nakamura et al. 2006).

Almost all VHL type 2 patients (high risk of PCC) harbour *VHL* missense mutations, whereas null *VHL* alleles cause type 1 disease (low risk of PCC). This suggests that complete loss of VHL protein function is incompatible with PCC development or that PCC reflects a gain-of-function mutant VHL protein (Nakamura et al. 2006). As complete loss of *VHL* gene in PCC is thought to be a very rare event, we can speculate that probably the absent amplification of *VHL* gene was due to poor tissue quality.

This series thus comprised 10 mutated and 47 non-mutated PCC patients, which means that in 18% patients the cause for tumour development was an inherited genetic mutation. Genotype-phenotype correlations showed that mutated patients were more frequently males, comparing with non-mutated cases that were mostly female patients. Also, mutated patients had younger age at diagnosis than non-mutated ones, even though NF1 patients had higher mean age at diagnosis than other mutated patients. The VHL patient had the lowest age at diagnosis.

It is known that tumours among patients with *VHL*, *SDHB* and *SDHD* mutations have lower ages at diagnosis compared with those with MEN2 and NF1 syndromes (Eisenhofer et al. 2011). In fact, in our findings, VHL patient had the lowest age and NF1 patients had the higher mean age at diagnosis. Periodic screening for germline mutations related to PCC/PGL's contributes to the younger age at diagnosis, both for patients with familial history or with sporadic disease. (Eisenhofer et al. 2011) As *NF1* gene has no hotspot for genetic screening, clinical features analysis might delay diagnose of this patients resulting in higher ages at diagnosis.

Differences in the age at diagnosis are also associated with different catecholamine phenotypes of tumours. Epinephrine-producing tumours (MEN2 and NF1) are associated with later ages at diagnosis than those that do not produce epinephrine (SDH and VHL). This can be explained by the differences in signs and symptoms caused by different catecholamine phenotypes. Other explanation is that tumourigenic processes for PCC/PGL development involve blocked differentiation of chromaffin progenitor cells during embryonic development and failure of neuronal apoptosis. Epinephrine-producing chromaffin cells develop later in embryogenesis and thus, tumourigenic processes associated with fully differentiated epinephrine-producing chromaffin cells occur later in embryogenesis or even after birth, leading to later onset of PCC/PGL; in contrast, tumourigenic processes associated with chromaffin progenitors lacking epinephrine production initiate earlier in embryogenesis, resulting in earlier onset of disease (Eisenhofer et al. 2011).

In contrast, SDH patients had higher mean age at diagnosis than MEN2 patients, which is not in agreement with previous reports. Earlier ages at diagnosis by MEN2 patients might be explained by the presence of bilateral tumours compared with SDH patients with unilateral PCC's. Multifocal tumours origin can be explained by single tumour stem cells occurring during embryogenesis and resulting in an earlier onset of PCC than those normally occurring later in life (Eisenhofer et al. 2011).

We have also observed that bilateral tumours developed only in mutated patients, specifically in MEN2 cases and in one NF1 patient. According to several reports, indeed familial PCC/PGL's are more likely to develop bilateral tumours and several reports indicate that MEN2 associated PCC's are more often bilateral (50%); NF1 patients develop bilateral PCC's more rarely (14%) (Welander et al. 2011; Young et al. 2012).

Mean tumour size was higher for non-mutated cases, but not with significant differences compared with mutated cases. Mean tumour size for both groups relies in small PCC's category, as it was defined that tumours below 6cm are considered small sized and those above or equal 6cm are large tumours (Agarwal et al. 2010). In addition, some reports defined that PCC's below 6cm are considered probable benign and those above or equal to 6cm are probable malignant tumours (Goldstein et al. 1999). In our series, patients with metastasis had a mean tumour size of 9cm, which supports this theory. However, increased size alone, although suggestive for malignancy, is not a good predictor of differences in biological behaviour of tumours (Agarwal et al. 2010).

Among the mutated tumours the largest ones were seen in NF1 patients and the smallest was the VHL tumour. In fact, NF1 protein is implicated in the GTPase activity to convert Ras into its inactive form, inhibiting Ras/Raf/MAPK signalling pathway, thus suppressing cell growth; on the other hand, mutant protein does not activate Ras which becomes constitutively activated to trigger the subsequent oncogenic downstream pathways (MAPK, PI3K and mTOR) responsible for cell proliferation and differentiation, thus leading to uncontrolled cell proliferation (Boyd et al. 2009; Welander et al. 2012). This mechanism could explain the large size of NF1 tumours comparing to SDH, MEN2 or VHL tumours.

Metastasis appeared only in non-mutated cases and relapse in only one mutated patient, contrarily to what was expected, as mutated patients have more predisposition to develop malignant tumours. Mutations in the *SDHB* gene are the most frequently associated with metastatic PCC/PGL's, predicting tumour aggressiveness and poor survival of patients, although a causal link has not been established yet. Nevertheless, the majority of patients with metastatic tumours do not carry mutations in any gene associated with PCC/PGL and our series of PCC's supports this. Sporadic PCC's could display increased angiogenesis, hypoxia and extracellular matrix elements in addition to

suppression of oxireductase enzymes and increased intracellular HIF concentrations that might explain malignancy (Ayala-Ramirez et al. 2011).

Both mutated and non-mutated patients had a mean PASS score of 4, indicating a possible aggressive behaviour of these tumours and suggesting that in this series PASS score probably is not a good predictor to associate malignancy with mutations.

Finally, microvessel density was smaller for mutated cases comparing with non-mutated. Microvessel density has been suggested to be a predictor of metastatic disease and to associate with tumour aggressiveness; this is reflected in our series, as mutated patients had non-metastatic tumours and lower microvessel density.

Metastatic tumours have been found to display a higher number of vessels compared with non-metastatic tumours (Weidner 1995). Among the angiogenic factors, vascular endothelial growth factor (VEGF) is a particular regulator of angiogenesis that is secreted by tumour cells. VEGF gene expression had previously been reported in PC12 pheochromocytoma cells and may be useful as a prognostic marker since its levels help distinguish benign from malignant tumours. In fact, higher VEGF expression and microvessel density was found in malignant compared with benign human PCC's (Zielke et al. 2002).

The second part of this study involved the screening of *SDH* mutations in 12 genomic DNA samples from PCC/PGL patients. In this series, six cases carried five different pathogenic mutations underlying PCC/PGL development, four of them in *SDH* genes and one in *MAX* gene, resulting in 50% patients whose cause for tumour development was an inherited genetic mutation.

Two patients were found to carry a large deletion in *SDHB* gene encompassing exon 1 and promoter (c.1-10413_73-3866del) which presumably leads to loss of the promoter or an abnormal protein due to alternative start codon. This alteration had already been identified by Cascón et al. in patients from the northwest of the Iberian Peninsula. The authors proposed a founder effect for this alteration, since all the patients showed a conserved breakpoint and conserved SNP flanking the deletion (Cascón et al. 2008). Notably, of the five families, one was from Porto, Portugal, a second was a Brazilian family and the remaining three were of Spanish origin, more specifically from Galicia,

close to the border with northern Portugal (Cascón et al. 2008). Recently, 11 Portuguese patients have also been identified with this large deletion. Results of this study showed that the deletion breakpoint matches the previously found by Cascón et al. and that the deleted allele from all 11 patients shares the same haplotype, evidencing a common origin of this pathogenic mutation carried by northern Portuguese patients.

The second mutation, a missense mutation in exon 1 of *SDHB* gene (c.32G>A, p. R11H), had been previously described by Ricketts et al. in a patient with renal cell carcinoma with no familial or personal history of PCC/PGL (Ricketts et al. 2008); however, the patient here described does not have signs of disease other than PGL, raising the question of what are the reasons behind the varying expressivity of *SDHB* mutations in different patients. In fact, several tumours have been associated with *SDHB* mutations: PCC and PGL are the most common, but also gastrointestinal stromal tumours (GIST), breast and papillary thyroid carcinoma (PTC), neuroblastoma, renal cell carcinoma (clear-cell and papillary RCC) and Carney-Stratakis syndrome (dyad of gastrointestinal stromal tumours and PGL) are also found in *SDHB* mutated patients (Bardella et al. 2011).

The frameshift mutation in exon 6 of *SDHB* gene (c.591 del.C; p.S198AfsX22) had been previously reported by Astuti et al. in a sporadic PCC patient (Astuti et al. 2001). Subsequent studies by Gimenez-Roqueplo, Benn and Fakhry found the same mutation in PGL patients (Gimenez-Roqueplo et al. 2003; Benn et al. 2006; Fakhry et al. 2007). The outcome of the mutation is a complete loss of mitochondrial complex II enzymatic activity, as further molecular and enzymatic analysis showed abnormal succinate cytochrome *c* reductase and quinol cytochrome *c* reductase activity. (Gimenez-Roqueplo et al. 2003)

The missense mutation in exon 1 of *SDHD* gene (c.3 G>C; p.M1I) was firstly reported by Badenhop et al. in a familial carotid body PGL (Badenhop et al. 2001). This mutation affected the first codon, blocking the initial methionine translation and thus protein formation.

Additionally, two non-pathogenic variants were identified - c.18 C>A; p.A6A and c.487 T>C; p.S163P -, both in *SDHB* gene constituting single nucleotide polymorphisms already reported by Cascón et al. and with a frequency of 4% and 2-3%, respectively, in the European population (Cascón et al. 2002). In this study, the frequency for the first SNP in exon 1 was 17% and for the second SNP in exon 5 was 8%, both higher than European frequencies.

Among *SDH* genes, *SDHB* and *SDHD* are known to be the most mutated ones and this series supports this fact.

Genotype-phenotype correlations showed that *SDH* mutated cases comprised more male than female patients and more PGL's than PCC's. *SDH* mutated patients often present more PGL's than PCC's, as it was also shown in this series. Mean age at diagnosis was lower in mutated patients compared to non-mutated patients, as expected, since mutations predispose to earlier formation of tumours. One malignant tumour was identified as carrying a *SDHB* large deletion, supporting the fact that *SDHB* has high potential of malignancy compared to other *SDH* genes.

The mechanisms behind the tumourigenesis induced by *SDH* mutations are still under study. It is known that the mitochondrial enzyme complex – SDH - is the only one involved both in the electron transport chain and in the Krebs cycle (Horsefield et al. 2006; Fishbein et al. 2012). The main functions of SDH enzyme include catalyzing the oxidation of succinate to fumarate in the Krebs cycle and also electrons transfer to the terminal acceptor ubiquinone or coenzyme Q in the electron transport chain, reducing it to ubiquinol (Horsefield et al. 2006; Welander et al. 2011; Fishbein et al. 2012).

Inactivation of SDH complex subunits has been found to result in the accumulation of succinate and also an increased production of oxygen free radicals (Karasek et al. 2010). Accumulated succinate moves out from the mitochondria, through the dicarboxylate carrier, to the cytosol, where it is able to inhibit the activity of HIFα prolyl hydroxylases (PHD's) like EglN1 and EglN3, causing a pseudo-hypoxic phenomenon (Smith et al. 2007). In normoxic conditions, PHD's hydroxylate two proline residues on HIFα, using α-ketoglutarate and oxygen as substrate and iron and ascorbate as co-factors. Thus, HIFα is recognized and targeted by VHL protein to oxygen-dependent ubiquitination by an ubiquitin ligase complex and consequently degraded in the proteasomal system (Bardella et al. 2011). However, when high levels of succinate inhibit PHD's, HIFα is not hydroxylated and subsequently escapes proteasomal degradation. This event leads to migration of HIFα from the cytosol to the nucleus, where it forms a heterodimer with HIFβ, causing an induction of expression of genes responsible for glycolysis, angiogenesis, proliferation and cell survival (Bardella et al. 2011); this phenomenon, where HIF is stabilized even in normoxia is termed pseudo-hypoxia. Numerous studies support this mechanism of tumourigenesis for PCC/PGL's development. In fact, angiogenic profile of

the tumours has been seen in complete loss of complex II function due to HIF deregulation (Bardella et al. 2011).

Another explanation for tumourigenesis caused by loss of function of SDH enzyme is the production of reactive oxygen species (ROS) in the electron transport chain. Although complex II is not a major local for ROS production, *SDH* mutations induce oxidative stress, genomic instability and tumourigenesis. Studies in model organisms, like mouse, worm and yeast showed that mutations affecting the ability to catalyze the electron transport from succinate to ubiquinone lead to electrons leakage and oxygen hypersensitivity phenotype with consequent elevation in oxidative stress, increased levels of ROS production and tumour growth. ROS can inhibit PHD's activity by oxidation of co-factors like ferrous iron and ascorbate resulting in a pseudo-hypoxic response (Bardella et al. 2011). The hypothesis of ROS being the link between *SDH* mutations and tumour development is under debate, since some studies showed no increased ROS production upon SDHB inactivation, but only an up-regulation of HIF-1α and HIF-2α. However, several other reports showed increased ROS production upon *SDH* mutations. The contrasting observations regarding ROS production, due to biological or technical reasons is to be determined (Bardella et al. 2011).

The third hypothesis for PCC/PGL's formation is a reduced apoptosis of sympathetic neuronal cells that carry *SDH* mutations, during embryogenesis. This apoptotic process is mediated by prolyl hydroxylase PHD3, which is inhibited due to succinate accumulation (Bardella et al. 2011).

Another supported theory is that accumulated succinate might inhibit other components of the α-ketoglutarate-dependent dioxygenase family, besides the PHD's and resulting in cell transformation and tumourigenesis. This family comprises enzymes involved in several biological roles, such as histone and nucleic acid demethylation. Histone demethylases inhibition can result in altered expression of oncogenes and tumour suppressor genes, such as those responsible for DNA repair, induction of apoptosis and growth inhibition (Bardella et al. 2011). As histone demethylases regulate some genes by recruitment of specific proteins, they have an impact in chromatin structure and gene expression and therefore, an epigenetic event might be specific for PCC/PGL's syndrome (Bardella et al. 2011). Succinate can thus act as messenger between mitochondria and cytosol as well as signal between mitochondria and nucleus, regulating gene expression (Bardella et al. 2011).

Finally, the G-protein-coupled GPR91 receptor might also be involved in the PCC/PGL's tumourigenic process. It was shown that succinate can act as ligand for GPR91 in the Krebs cycle, resulting in unexpected signalling functions, such as mediating vessel growth through the release of proangiogenic factors (like VEGF). Accumulated succinate leads to the deregulation of GPR91 causing sustainment of signalling pathway and stimulation of endothelial cells proliferation. Thus, altered levels of succinate, through the activation of its receptor GPR91, can promote tumour onset and progression (Bardella et al. 2011).

The induction of tumourigenesis triggered by *SDH* mutations represents an example of the importance of metabolic alterations in tumour development. Regarding cancer metabolism, a well-known feature is the "Warburg Effect" where malignant cells use aerobic glycolysis to generate energy instead of mitochondrial oxidative phosphorylation like normal cells (López-Lázaro 2008; Welander et al. 2011). Hypoxic conditions observed in tumours can exacerbate the upregulation of glucose transporters and other multiple enzymes of the glycolytic pathway. Therefore, hypoxia can raise the HIF1α and HIF2α transcription factors levels, independently, which then upregulate glycolysis, leading to active cell proliferation (Hanahan et al. 2011; Fliedner et al. 2012).

Mutations of SDH subunits have been shown to cause activation of hypoxic response, even in normoxic conditions, a process referred as pseudo-hypoxia. Tumourigenesis is also associated with glycolytic phenotype of tumours, since the Warburg effect takes place when *SDH* mutations lead to impaired oxidative phosphorylation and Krebs cycle in PCC/PGL's. First, electron transport chain by complex II is impaired in oxidative phosphorylation and second, compromised conversion of succinate to fumarate leads to increased amounts of succinate which inhibits PHD's and causes HIF's activation. HIF-responsive genes are then activated reinforcing glycolysis, while oxidative phosphorylation is decreased (Favier et al. 2009; Fliedner et al. 2012).

Recently, Rapizzi et al. studied a series of 57 PGL patients to evaluate SDH activity and expression and mitochondrial homeostasis (Rapizzi et al. 2012). It was the first systematic study in PGL's about SDH activity and expression, only reported so far in a patient with *SDHD* mutation and two cases with *SDHB* and *SDHA* mutations that result in lack of SDH activity, as well as in 22 head and neck PGL's (Gimenez-Roqueplo et al. 2001; Dekker et al. 2003; Burnichon et al. 2010). The results showed 19

germline mutations (ten *SDHD*, three *SDHB*, one *SDHC*, two *RET*, one *VHL* and two *MAX*) and two somatic mutations (*SDHD*) among the PGL cases. All *SDH*-mutated tumours displayed decreased SDH activity, as well as one case of *MAX*-mutated PGL and two wild-type cases at peripheral DNA genetic analysis that were then found to have *SDHD* somatic mutations (p.Asp113MetfsX21 already described by Cascón et al. and a novel mutation p.Leu7CysfsX7). *RET* and *VHL* mutated tumours displayed normal SDH activity. LOH was found only in 70% of the patients with a decreased SDH activity and expression (Rapizzi et al. 2012).

RT-PCR study on the four SDH subunit transcript levels was performed to discover the mechanism underlying decreased SDH activity and expression in one of the two *MAX*-mutated tumours (p.Leu94Pro). In the *MAX*-mutated tissue, the transcript levels of SDHB, SDHC, and SDHD were slightly increased compared with control; the authors suggested that SDH complex instability and disassembling rather than a negative transcriptional regulation were involved in SDH decreased expression in the *MAX*-mutated tissue (Rapizzi et al. 2012).

To address the differences in SDH activity and expression, the authors analysed citrate synthase (CS) activity, an enzyme present in the mitochondrial matrix and involved in the TCA cycle, voltage-dependent anion channel (VDAC) expression, a protein located on mitochondria outer membrane, and cytochrome c oxidase (COX) activity, an enzyme of the respiratory chain located in the inner mitochondrial membrane as mitochondrial complex IV. The results showed normal activity for CS and VDAC elements and extreme variability of COX activity among the different tissue samples. Thus, CS analysis suggested that enzyme function within the mitochondrial matrix was not impaired by the presence of *SDH* mutations. VDAC and COX were investigated, since preliminary data on *SDH*-mutated PGL's suggested a structural alteration of mitochondrial cristae. These observations suggested that in these tumours, neither the outer mitochondrial membrane, as shown by the normal VDAC expression, nor the mitochondrial matrix, as shown by the normal CS activity were modified and only the mitochondrial cristae were altered, as seen by COX activity (Rapizzi et al. 2012).

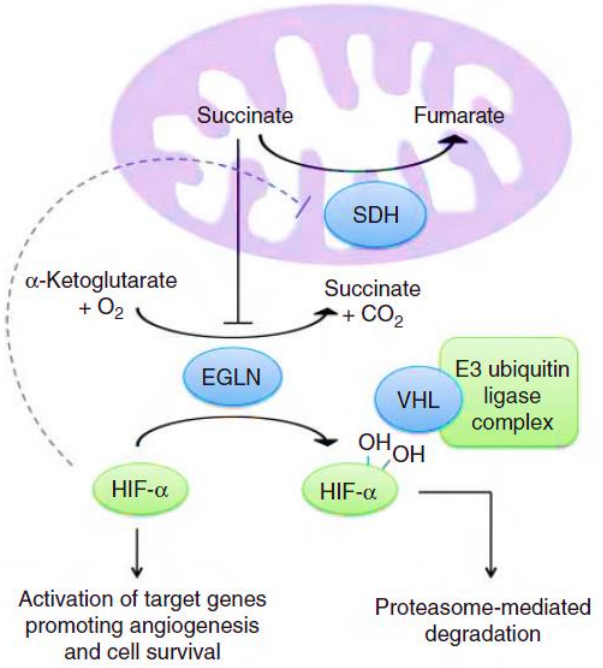


Figure 20 - Regulation of HIF. Inactivation of SDH, VHL, or EGLN1 are believed to cause a pseudo-hypoxic response where HIF-1α and/or HIF-2α escape ubiquitination and are allowed to accumulate (Welander et al. 2011).

SDHAF2 gene was also screened in the Polish PCC series, with no mutations found. Nevertheless, mutations in this gene are also associated with alterations in SDH complex.

In 1981 van Baars et al. described a Dutch family with a single nucleotide c.232G>A change in exon 2, which caused G78R mutation in the most conserved region of the protein (Baars et al. 1981).

Subsequently studies using affected individuals from different branches of the previously described Dutch PGL2 lineage identified the same mutation in *SDHAF2* gene (Hao et al. 2009; Kunst et al. 2011).

Flavination of SDHA had been proposed to require at least one additional protein, SDHAF2, which participates in the FAD attachment. SDHA flavination was decreased by 95% in tumours from three patients with PGL in comparison with control tumours from two sporadic PGL patients. Thus, G78R mutation destabilizes SDHAF2 and impairs its interaction with SDHA (Hao et al. 2009).

Loss of SDHAF2 results in absence of SDH function and a reduction in the stability of the SDH complex, leading to reduced levels of all subunits (Kunst et al. 2011).

In the first part of this thesis, we aimed to identify mutations in the new susceptibility genes for PCC - *MAX* and *TMEM127* - in patients without SDH, MEN2, VHL or NF1 syndromes. The genetic analysis of *MAX* and *TMEM127* was difficult, with the appearance of false positive cases for pathogenic mutations in these two genes, possibly due to poor quality material.

In the second part of the thesis, time constraints did not allow us to screen for *MAX* and *TMEM127* mutations in cases without *SDH* mutations. However, a PCC case belonging to a young patient with familial PCC history prompted us to screen for mutations in *MAX* gene, with consequent identification of a pathogenic nonsense mutation (c.97C>T; p.R33X). This nonsense mutation in *MAX* gene was reported by Comino-Mendez et al., which confirmed the lack of full-length MAX in the tumour carrying this genetic variant, by immunohistochemical analysis (Comino-Méndez et al. 2011).

Although our series showed only one pathogenic mutation in the *MAX* gene, several reports identified *MAX* and *TMEM127* genes as possible candidates for PCC pathogenesis.

Comino-Mendez *et al.* used next-generation sequencing for *MAX* gene to analyse three independent familial PCC cases without a germline mutation in any known susceptibility gene. Three alterations were found and these PCC's showed MAX protein loss of expression by immunohistochemistry and LOH by tumour DNA analysis. LOH in *MAX* gene was discovered to be caused by uniparental disomy (UPD) from paternal origin with loss of the maternal allele. In addition, paternal origin of the mutated allele was also detected in other six families, showing a preferentially paternal transmission of the disease (Comino-Méndez et al. 2011).

More recently, a large series of 1694 unrelated PCC/PGL's without evident germline mutations in *RET*, *VHL*, *SDHB*, *SDHC*, *SDHD*, and *TMEM127* genes was screened for *MAX* gene. Results showed 23 patients carrying 16 different heterozygous variants. Further 245 tumours were analysed and four somatic mutations were identified. Among these, 18 were novel variants, five of them disrupting MAX protein, 11 nontruncating variants, seven of them changing conserved or highly conserved amino acids located within the basic helixloop-helix leucine zipper (bHLH-Zip) domain of the MAX protein and four predicted as benign (Burnichon et al. 2012).

Immunohistochemical analysis showed loss of MAX expression for all tumours carrying truncating mutations and positive protein staining in all nontruncating variants. LOH of the *MAX* wild-type allele

was found in 16 of 18 tumours. This study identified pathogenic germline *MAX* variants in 1% of the 1694 cases, mostly in PCC patients but also in 3 PGL cases. Variants were more frequent in exons 3 and 4, which comprise some of the most important residues within the conserved bHLH-Zip domain of MAX (Burnichon et al. 2012).

Most of the mutations identified so far, affected highly conserved amino acids within the basic helix-loop-helix leucine zipper domain of MAX, the structural element for specificity and stability of homodimer and heterodimer formation, as well as DNA recognition. This domain is implicated in protein-protein interactions and DNA binding; alterations that affect bHLHZip domain could lead to impaired MAX function and tumour development (Cascón et al. 2012).

Ribon et al. showed that MAX functions as regulator of MYC-dependent transcriptional activation. In the PC12 cells (derived from rat adrenal PCC), that have a homozygous alteration in the *MAX* gene, the encoded protein is uncapable of forming homodimers or heterodimers leading to the inhibition of transcriptional repression from E-box elements; reintroducing MAX in these cells caused transcriptional repression and reduced growth rate (Ribon et al. 1994). These findings also demonstrated that MYC functions in the absence of normally functioning MAX protein, proved later by Gallant et al. in *Drosophila* and leading to the conclusion that MAX is related more to repression than to activation (Gallant et al. 2009).

MAX tumours, when compared with PCC/PGL's with mutations in other genes, showed significant enhancement of mTOR pathway components. This finding revealed that *MAX* mutations not only deregulate MYC function but also lead to altered mTOR pathway and PCC/PGL's development (Cascón et al. 2012). In fact, PI3K/AKT/mTOR and MYC/MAX/MXD1 pathways were recently associated, since phosphorylated S6K1, activated by mTOR to regulate cell growth, inhibits the tumour suppressor function of MAD1, leading to deregulation of MYC, cell growth and activation of proliferation (Zhu et al. 2008).

In addition to *MAX*, Qin et al. identified *TMEM127* gene as a novel PCC susceptibility gene. Sequencing 102 PCC cases, 19 with familial history and 83 as sporadic cases, seven germline mutations were detected. Four were detected in 12 families and the other three were identified

among the sporadic cases, all of them without mutations in other PCC susceptibility genes. Six mutations were splice-site or nonsense mutations and were associated with interruption of protein reading frame. Also, LOH was proved to occur at *TMEM127* locus with loss of the wild type allele leading to the conclusion of *TMEM127* being a classic tumour suppressor gene (Qin et al. 2010).

In a subsequent study Qin et al. considered a group of 990 samples from PCC/PGL patients without mutations in other susceptibility genes to analyse possible germline mutations in *TMEM127* gene. Thirteen novel changes were found and six variants previously reported comprising small deletions, duplications, insertions, nonsense mutations, splice site substitutions, nonsynonymous missense mutations and substitutions in the UTR region, all of them potentially pathogenic. Also, LOH was detected in four cases and no large deletions were found among 545 samples analysed. So, point mutations and small deletions/insertions are the main cause of TMEM127 deregulation, not excluding other hypotheses such as gene promoter methylation (Yao et al. 2012).

All patients with *TMEM127* mutations developed PCC, one-third of them with bilateral tumours; no PGL's were found so far in *TMEM127* mutated patients. The age of onset of mutated tumours was similar to patients with sporadic disease, which does not follows the earlier manifestation seen in hereditary PCC's caused by mutations in other susceptibility genes. Only one patient carrying a missense mutation had metastases (Yao et al. 2012).

Four novel truncating mutations, two of them nonsense and two other frameshift mutations were discovered by Abermil *et al.* in a French series of 642 unrelated patients with PCC/PGL. All mutated patients had PCC and secreted high levels of metanephrine, clustering TMEM127 tumours with epinephrine producing tumours like RET or NF1 tumours. Once more, no large deletions or duplications were detected in *TMEM127*, indicating that genetic testing should be restricted to direct sequencing (Abermil et al. 2012).

Abermil et al. combined their data with Yao et al. and Neumann et al. studies, concluding that *TMEM127* mutations had a prevalence of 2% among the 1676 patients reported until now (Abermil et al. 2012).

TMEM127 gene in humans is expressed both in normal tissue and also in many cancer cell lines. Colocalization studies by Qin *et al.* showed that TMEM127 is present in vesicular organelles like

endosomes, Golgi and lysosomes. Also, TMEM127 was associated with the plasma membrane, mostly when endocytosis is inhibited and an increasing pH causes TMEM127 trapping in endosomal structures. So, this gene was linked with protein trafficking between plasma membrane and endomembrane organelles, like Golgi and lysosomes (Qin et al. 2010). Green fluorescent protein expression in a subsequent study showed diffuse distribution of mutated *TMEM127* in the cytoplasm, different from the wild-type which is located mostly in plasma membrane and endomembrane organelles. This localization of mutated proteins can be associated with mTOR accessibility to its regulators (Yao et al. 2012).

Qin *et al.* showed increased phosphorylation of 4EBP1 in some cell lines with *TMEM127* knockdown and cells were larger and proliferative, features consistent with mTORC1 functions. Also, *TMEM127* overexpression was associated with reduced mTORC1 signalling and decreased cell proliferation. Moreover, increased phosphorylation of S6K in lysates from *TMEM127* mutant PCC was detected. To determine a possible influence of TMEM127 in mTOR subcellular localization, confocal microscopy was used and showed the same intracellular domain occupied both by TMEM127 and active mTOR (Qin et al. 2010).

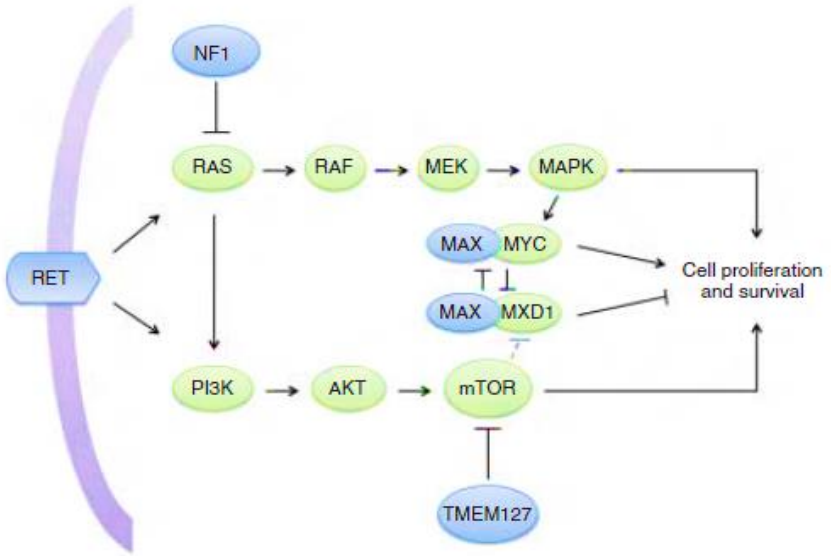


Figure 21 – Signalling pathways putatively involved in PCC/PGL's development. Activation of mTOR may constitute a mechanism for tumour development caused by mutations in *RET*, *NF1*, or *TMEM127*; MAX may control cell proliferation by forming dimers with MXD1 that antagonize the transcriptional activity of MYC (Welander et al. 2011).

Summarizing, *VHL* and *SDH* related PCC/PGL's share a similar gene expression profile linked to hypoxia and angiogenesis, where a stabilization of HIF-1α and/or HIF-2α, under normoxic conditions, may play a central role in the pathogenesis. In contrast, *RET*, *NF1*, *TMEM127* and *MAX* related PCC/PGL's display a profile that can be linked to an activation of kinase signalling pathways, such as RAS/RAF/MAPK, PI3K/AKT and mTOR signalling pathways, promoting cell proliferation, growth and survival.

7. CONCLUSIONS

In this study, the aim was to identify the genetic defects in *SDH*, *SDHAF2*, *MAX*, *TMEM127*, *RET* and *VHL* that underlie the development of tumours of the paraganglionic tissue, termed pheochromocytomas and paragangliomas.

The results in a series of 57 PCC patients revealed three cases with absence of SDH expression, but only one case with a detected mutation. The existence of mutations in other recently identified PCC/PGL susceptibility genes prompted the study of *MAX* and *TMEM127* in cases without evidence of syndromic disease such as MEN2, NF1 or VHL – although no mutations were identified, the involvement of these genes cannot be excluded, since detection of large deletions was not performed. Concomitantly, expression studies for MAX and TMEM127 proteins were not executed and thus, despite of absent mutations, some PCC's might had loss of protein expression caused by alterations that were not investigated in this study.

The results in a series of 12 PCC/PGL patients revealed four cases with *SDHB* mutations, one case with a *SDHD* mutation and one case with a *MAX* mutation. *MAX* screening was only performed for one case with familial history and *TMEM127* was not screened. Among the six non-mutated cases some might carry *MAX* or *TMEM127* mutations that were not assessed.

Genetic testing is proved to be the best approach in PCC/PGL diagnose, as ten known susceptibility genes have been identified so far, making these tumours more commonly associated with an inherited mutation than any other cancer type. Nevertheless, additional susceptibility genes probably remain to be discovered, as some families with PCC or PGL did not have an identified genetic cause and some apparently sporadic cases had young age at diagnosis and other features that point to inherited genetic alterations.

Knowledge of the clinical features linked to different hereditary backgrounds can be crucial for decision making regarding treatment and surveillance. Much more learning about the germline and somatic genetics in PCC/PGL and insights into the biology of these tumours is essential to provide targets for therapeutics.

8. REFERENCES

Abermil, N., M. Guillaud-Bataille, N. Burnichon, A. Venisse, P. Manivet, L. Guignat, D. Drui, M. Chupin, C. Josseaume, H. Affres, P.-F. Plouin, J. Bertherat, X. Jeunemaître and A.-P. Gimenez-Roqueplo (2012). "**TMEM127 Screening in a Large Cohort of Patients with Pheochromocytoma and/or Paraganglioma**." *The Journal of Clinical Endocrinology & Metabolism* **97**: 805-809.

Ackrell, B. A. C. (2000). "**Progress in understanding structure-function relationships in respiratory chain complex II**." *FEBS Letters* **466**: 1-5.

Agarwal, A., P. K. Mehrotra, M. Jain, S. K. Gupta, A. Mishra, G. Chand, G. Agarwal, A. K. Verma, S. K. Mishra and U. Singh (2010). "**Size of the Tumor and Pheochromocytoma of the Adrenal Gland Scaled Score (PASS): Can They Predict Malignancy?**" *World Journal of Surgery*.

Alberts, B., A. Johnson and J. Lewis (2002). **Molecular Biology of the Cell**, New York: Garland Science.

Astuti, D., F. Latif, A. Dallol, P. L. M. Dahia, F. Douglas, E. George, F. Sköldberg, E. S. Husebye, C. Eng and E. R. Maher (2001). "**Gene Mutations in the Succinate Dehydrogenase Subunit SDHB Cause Susceptibility to Familial Pheochromocytoma and to Familial Paraganglioma**." *The American Journal of Human Genetics* **69**: 49-54.

Ayala-Ramirez, M., L. Feng, M. M. Johnson, S. Ejaz, M. A. Habra, T. Rich, N. Busaidy, G. J. Cote, N. Perrier, A. Phan, S. Patel, S. Waguespack and C. Jimenez (2011). "**Clinical Risk Factors for Malignancy and Overall Survival in Patients with Pheochromocytomas and Sympathetic Paragangliomas: Primary Tumor Size and Primary Tumor Location as Prognostic Indicators**." *The Journal of Clinical Endocrinology & Metabolism* **96**: 717-725.

Baars, F. v., P. v. d. Broek, C. Cremers and J. Veldman (1981). "**Familial non-chromaffinic paragangliomas (glomus tumors) : clinical aspects.**" *Laryngoscope* **91**: 988-996.

Badenhop, R. F., S. Cherian, R. S. Lord, B. E. Baysal, P. E. Taschner and P. R. Schofield (2001). "**Novel mutations in the SDHD gene in pedigrees with familial carotid body paraganglioma and sensorineural hearing loss.**" *Genes Chromosomes Cancer* **31**: 255-263.

Bardella, C., P. J. Pollard and I. Tomlinson (2011). "**SDH mutations in cancer**." *Biochimica et Biophysica Acta* **1807**: 1432-1443.

Bayley, J.-P., H. P. M. Kunst, A. Cascon, M. L. Sampietro, J. Gaal, E. Korpershoek, A. Hinojar-Gutierrez, H. J. L. M. Timmers, L. H. Hoefsloot, M. A. Hermsen, C. Suárez, A. K. Hussain, A. H. J. T. Vriends, F. J. Hes, J. C. Jansen, C. M. Tops, E. P. Corssmit, P. d. Knijff, J. W. M. Lenders, C. W. R. J. Cremers, P. Devilee, W. N. M. Dinjens, R. R. d. Krijger and M. Robledo (2010). "**SDHAF2 mutations in familial and sporadic paraganglioma and phaeochromocytoma**." *The Lancet Oncology* **11**: 366–372.

Baysal, B., J. Willett-Brozick, P. Filho, E. Lawrence, E. Myers and R. Ferrell (2004). "**An Alu-mediated partial SDHC deletion causes familial and sporadic paraganglioma**." *Journal of Medical Genetics* **41**: 703–709.

Baysal, B. E., J. E. Willett-Brozick, E. C. Lawrence, C. M. Drovdlic, S. A. Savul, D. R. McLeod, H. A. Yee, D. E. Brackmann, W. H. Slattery, E. N. Myers, R. E. Ferrell and W. S. Rubinstein (2002). "**Prevalence of SDHB, SDHC, and SDHD germline mutations in clinic patients with head and neck paragangliomas.**" *Journal of Medical Genetics* **39**: 178-183.

Benn, D. E., A.-P. Gimenez-Roqueplo, J. R. Reilly, J. Bertherat, J. Burgess, K. Byth, M. Croxson, P. L. M. Dahia, M. Elston, O. Gimm, D. Henley, P. Herman, V. Murday, P. Niccoli-Sire, J. L. Pasieka, V. Rohmer, K. Tucker, X. Jeunemaitre, D. J. Marsh, P.-F. Plouin and B. G. Robinson (2006). "**Clinical Presentation and Penetrance of Pheochromocytoma/Paraganglioma Syndromes**." *The Journal of Clinical Endocrinology & Metabolism* **91**: 827– 836.

Benn, D. E., A. L. Richardson, D. J. Marsh and B. G. Robinson (2006). "**Genetic Testing in Pheochromocytoma- and Paraganglioma-Associated Syndromes**." *New York Academy of Sciences* **1073**: 104-111.

Blake, M. A. and A. T. Sweeney (2011) **Pheochromocytoma**.

Boyd, K. P., B. R. Korf and A. Theos (2009). "**Neurofibromatosis type 1**." *Journal of the American Academy of Dermatology* **61**: 1-16.

Burnichon, N., J.-J. Brière, R. Libé, L. Vescovo, J. Rivière, F. Tissier, E. Jouanno, X. Jeunemaitre, P. Bénit, A. Tzagoloff, P. Rustin, J. Bertherat, J. Favier and A.-P. Gimenez-Roqueplo (2010). "**SDHA is a tumor suppressor gene causing paraganglioma**." *Human Molecular Genetics* **19**: 3011-3020.

Burnichon, N., A. Cascón, F. Schiavi, N. P. Morales, I. Comino-Méndez, N. Abermil, L. Inglada-Pérez, A. A. d. Cubas, L. Amar, M. Barontini, S. B. d. Quirós, J. Bertherat, Y.-J. Bignon, M. J. Blok, S. Bobisse, S. Borrego, M. Castellano, P. Chanson, M.-D. Chiara, E. P. M. Corssmit, M. Giacchè, R. R. d. Krijger, T. Ercolino, X. Girerd, E. B. Gómez-García, Á. Gómez-Graña, I. Guilhem, F. J. Hes, E. Honrado, E. Korpershoek, J. W. M. Lenders, R. Letón, A. R. Mensenkamp, A. Merlo, L. Mori, A. Murat, P. Pierre, P.-F. Plouin, T. Prodanov, M. Quesada-Charneco, N. Qin, E. Rapizzi, V. Raymond, N. Reisch, G. Roncador, M. Ruiz-Ferrer, F. Schillo, A. P. A. Stegmann, C. Suarez, E. Taschin, H. J. L. M. Timmers, C. M. J. Tops, M. Urioste, F. Beuschlein, K. Pacak, M. Mannelli, P. L. M. Dahia, G. Opocher, G. Eisenhofer, A.-P. Gimenez-Roqueplo and M. Robledo (2012). "**MAX Mutations Cause Hereditary and Sporadic Pheochromocytoma and Paraganglioma**." *Clinical Cancer Research* **18**: 2828-2837.

Carlson, K. M., S. Dou, D. Chi, N. Scavarda, K. Toshima, C. E. Jackson, S. A. J. Wells, P. J. Goodfellow and H. Donis-Keller (1994). "**Single missense mutation in the tyrosine kinase catalytic domain of the RET protooncogene is associated with multiple endocrine neoplasia type 2B.**" *Proceedings of the National Academy of Sciences* **91**: 1579-1583.

Cascón, A., A. Cebrián, S. Ruiz-Llorente, D. Tellería, J. Benítez and M. Robledo (2002). "**SDHB mutation analysis in familial and sporadic phaeochromocytoma identifies a novel mutation.**" *Journal of Medical Genetics* **39**.

Cascón, A., Í. Landa, E. López-Jiménez, A. Díez-Hernández, M. Buchta, C. Montero-Conde, S. Leskelä, L. J. Leandro-García, R. Letón, C. Rodríguez-Antona, C. Eng, H. P. H. Neumann and M. Robledo (2008). "**Molecular characterisation of a common SDHB deletion in paraganglioma patients**." *Journal of Medical Genetics* **45**: 233–238.

Cascón, A. and M. Robledo (2012). "**MAX and MYC: a heritable breakup.**" *Cancer Research* **72**: 3119-3124.

Comino-Méndez, I., F. J. Gracia-Aznárez, F. Schiavi, I. Landa, L. J. Leandro-García, R. Letón, E. Honrado, R. Ramos-Medina, D. Caronia, G. Pita, Á. Gómez-Graña, A. A. d. Cubas, L. Inglada-Pérez, A. Maliszewska, E. Taschin, S. Bobisse, G. Pica, P. Loli, R. Hernández-Lavado, J. A. Díaz, M. Gómez-Morales, A. González-Neira, G. Roncador, C. Rodríguez-Antona, J. Benítez, M. Mannelli, G. Opocher, M. Robledo and A. Cascón (2011). "**Exome sequencing identifies MAX mutations as a cause of hereditary pheochromocytoma**." *Nature Genetics* **43**: 663-667.

Cooper, G. M. (2000). **The Cell: A Molecular Approach**, Sunderland (MA): Sinauer Associates.

Dahia, P. L., K. Hao, J. Rogus, C. Colin, M. A. Pujana, K. Ross, D. Magoffin, N. Aronin, A. Cascon, C. Y. Hayashida, C. Li, S. P. Toledo and C. D. Stiles (2005). "**Novel pheochromocytoma susceptibility loci identified by integrative genomics.**" *Cancer Research* **65**: 9651-9658.

Dekker, P. B. D., P. C. Hogendoorn, N. Kuipers-Dijkshoorn, F. A. Prins, S. G. v. Duinen, P. E. Taschner, A. G. v. d. Mey and C. J. Cornelisse (2003). "**SDHD mutations in head and neck paragangliomas result in destabilization of complex II in the mitochondrial respiratory chain with loss of enzymatic activity and abnormal mitochondrial morphology.**" *The Journal of Pathology* **201**: 480-486.

Dekker, P. B. D., P. C. W. Hogendoorn, N. Kuipers-Dijkshoorn, F. A. Prins, S. G. v. Duinen, P. E. M. Taschner, A. G. L. v. d. Mey and C. J. Cornelisse (2003). "**SDHD mutations in head and neck paragangliomas result in destabilization of complex II in the mitochondrial respiratory chain with loss of enzymatic activity and abnormal mitochondrial morphology**." *The Journal of Pathology* **201**: 480-486.

DeLellis, R. A. (2004). **Who Classification of Tumors - Pathology and Genetics of Tumours of Endocrine Organs**.

Dudek, R. W. (2000). **High-Yield Histology**, Lippincott Williams & Wilkins.

Duff, S. E., C. Li, J. M. Garland and S. Kumar (2003). "**CD105 is important for angiogenesis: evidence and potential applications.**" *The FASEB Journal* **17**: 984-992.

Ecevit, O., M. A. Khan and D. J. Goss (2010). "**Kinetic Analysis of B/HLH/Z Transcription Factors MYC, MAX and MAD Interaction with Cognate DNA**." *Biochemistry* **49**: 2627–2635.

Eisenhofer, G., H. J. Timmers, J. W. M. Lenders, S. R. Bornstein, O. Tiebel, M. Mannelli, K. S. King, C. D. Vocke, W. M. Linehan, G. Bratslavsky and K. Pacak (2011). "**Age at Diagnosis of Pheochromocytoma Differs According to Catecholamine Phenotype and Tumor Location**." *The Journal of Clinical Endocrinology & Metabolism* **96**: 375–384.

Fakhry, N., P. Niccoli-Sire, A. Barlier-Seti, R. Giorgi, A. Giovanni and M. Zanaret (2007). "**Cervical paragangliomas: is SDH genetic analysis systematically required?**" *European Archives of Oto-Rhino-Laryngology* **265**: 557-563.

Favier, J., J.-J. Brière, N. Burnichon, J. Rivière, L. Vescovo, P. Benit, I. Giscos-Douriez, A. D. Reyniès, J. Bertherat, C. Badoual, F. Tissier, L. Amar, R. Libé, P.-F. Plouin, X. Jeunemaitre, P. Rustin and A.-P. Gimenez-Roqueplo (2009). "**The Warburg Effect Is Genetically Determined in Inherited Pheochromocytomas**." *Plos One* **4**.

Fishbein, L. and K. L. Nathanson (2012). "**Pheochromocytoma and paraganglioma: understanding the complexities of the genetic background**." *Cancer Genetics* **205**: 1-11.

Fliedner, S. M. J., N. Kaludercic, X.-S. Jiang, H. Hansikova, Z. Hajkova, J. Sladkova, A. Limpuangthip, P. S. Backlund, R. Wesley, L. Martiniova, I. Jochmanova, N. K. Lendvai, J. Breza, A. L. Yergey, N. Paolocci, A. S. Tischler, J. Zeman, F. D. Porter, H. Lehnert and K. Pacak (2012). "**Warburg Effect's Manifestation in Aggressive Pheochromocytomas and Paragangliomas: Insights from a Mouse Cell Model Applied to Human Tumor Tissue**." *Plos One* **7**.

Gallant, P. and D. Steiger (2009). "**Myc's secret life without Max**." *Cell Cycle* **8**: 3848 - 3853.

Gill, A. J., D. E. Benn, A. Chou, A. Clarkson, A. Muljono, G. Y. Meyer-Rochow, A. L. Richardson, S. B. Sidhu, B. G. Robinson and R. J. Clifton-Bligh (2010). "**Immunohistochemistry for SDHB triages genetic testing of SDHB, SDHC, and SDHD in paraganglioma-pheochromocytoma syndromes**." *Human Pathology* **41**: 805–814.

Gimenez-Roqueplo, A.-P., J. Favier, P. Rustin, J.-J. Mourad, P.-F. Plouin, P. Corvol, A. Rötig and X. Jeunemaitre (2001). "**The R22X Mutation of the SDHD Gene in Hereditary Paraganglioma Abolishes the Enzymatic Activity of Complex II in the Mitochondrial Respiratory Chain and Activates the Hypoxia Pathway**." *The American Journal of Human Genetics* **69**: 1186–1197.

Gimenez-Roqueplo, A.-P., J. Favier, P. Rustin, C. Rieubland, M. Crespin, V. Nau, P. K. V. Kien, P. Corvol, P.-F. Plouin and X. Jeunemaitre (2003). "**Mutations in the SDHB Gene Are Associated with Extra-adrenal and/or Malignant Phaeochromocytomas**." *Cancer Research* **63**: 5615–5621.

Gimenez-Roqueplo, A.-P., J. Favier, P. Rustin, C. Rieubland, V. Kerlan, P.-F. Plouin, A. Rötig and X. Jeunemaitre (2002). "**Functional Consequences of a SDHB Gene Mutation in an Apparently Sporadic Pheochromocytoma**." *The Journal of Clinical Endocrinology & Metabolism* **87**: 4771-4774.

Goldstein, R. E., J. A. O'Neill, G. W. Holcomb, W. M. Morgan, W. W. Neblett, J. A. Oates, N. Brown, J. Nadeau, B. Smith, D. L. Page, N. N. Abumrad and H. W. Scott (1999). "**Clinical Experience Over 48 Years With Pheochromocytoma**." *Annals of Surgery* **229**: 755-766.

Hanahan, D. and R. A. Weinberg (2011). "**Hallmarks of Cancer: The Next Generation**." *Cell* **144**: 646-674.

Hao, H.-X., O. Khalimonchuk, M. Schraders, N. Dephoure, J.-P. Bayley, H. Kunst, P. Devilee, C. W. R. J. Cremers, J. D. Schiffman, B. G. Bentz, S. P. Gygi, D. R. Winge, H. Kremer and J. Rutter (2009). "**SDH5, a Gene Required for Flavination of Succinate Dehydrogenase, Is Mutated in Paraganglioma**." *Science* **325**: 1139-1142.

Hayat, M. A. (2010). **Methods of Cancer Diagnosis, Therapy, and Prognosis**, Springer.

Hirawake, H., M. Taniwaki, A. Tamura, H. Amino, E. Tomitsuka and K. Kita (1999). "**Characterization of the human SDHD gene encoding the small subunit of cytochrome b (cybS) in mitochondrial succinate-ubiquinone oxidoreductase**." *Biochimica et Biophysica Acta* **1412**: 295-300.

Horsefield, R., V. Yankovskaya, G. Sexton, W. Whittingham, K. Shiomi, S. mura, B. Byrne, G. Cecchini and S. Iwata (2006). "**Structural and Computational Analysis of the Quinone-binding Site of Complex II (Succinate-Ubiquinone Oxidoreductase) - A Mechanism of electron transfer and proton conduction during ubiquinone reduction**." *Journal of Biological Chemistry* **281**: 7309-7316.

Iacobone, M., F. Schiavi, M. Bottussi, E. Taschin, S. Bobisse, A. Fassina, G. Opocher and G. Favia (2011). "**Is genetic screening indicated in apparently sporadic pheochromocytomas and paragangliomas?**" *Surgery* **150**: 1194-1201.

Jafri, M. and E. R. Maher (2012). "**The genetics of phaeochromocytoma: using clinical features to guide genetic testing**." *European Journal of Endocrinology* **166**: 151-158.

Janecke, A. R., J. E. Willett-Brozick, C. Karas, M. Hasipek, J. Loeffler-Ragg and B. E. Baysal (2010). "**Identification of a 4.9-kilo base-pair Alu-mediated founder SDHD deletion in two extended paraganglioma families from Austria**." *Journal of Human Genetics* **55**: 182–185.

Jiang, S. and P. L. M. Dahia (2011). "**Minireview: The Busy Road to Pheochromocytomas and Paragangliomas Has a New Member, TMEM127**." *Endocrinology* **152(6)**: 2133-2140.

Jubb, A. M., H. I. Hurwitz, W. Bai, E. B. Holmgren, P. Tobin, A. S. Guerrero, F. Kabbinavar, S. N. Holden, W. F. Novotny, G. D. Frantz, K. J. Hillan and H. Koeppen (2006). "**Impact of Vascular Endothelial Growth Factor-A Expression, Thrombospondin-2 Expression, and Microvessel Density on the Treatment Effect of Bevacizumab in Metastatic Colorectal Cancer**." *American Society of Clinical Oncology* **24**: 217-227.

Jung, I., S. Gurzu, M. Raica, A. M. Cîmpean and Z. Szentirmay (2009). "**The differences between the endothelial area marked with CD31 and CD105 in colorectal carcinomas by computer-assisted morphometrical analysis**." *Journal of Morphology and Embryology* **50**: 239–243.

Karasek, D., Z. Frysak and K. Pacak (2010). "**Genetic Testing for Pheochromocytoma**." *Current Hypertension Reports* **12**: 456 - 464.

Kay, S. M. and H. Flageole (2008) **Adrenal Glands**

Kim, W. Y. and W. G. Kaelin (2004). "**Role of VHL Gene Mutation in Human Cancer**." *Journal of Clinical Oncology* **22**: 4991-5004.

Korpershoek, E., J. Favier, J. Gaal, N. Burnichon, B. v. Gessel, L. Oudijk, C. Badoual, N. Gadessaud, A. Venisse, J.-P. Bayley, M. F. v. Dooren, W. W. d. Herder, F. Tissier, P.-F. Plouin, F. H. v. Nederveen, W. N. M. Dinjens, A.-P. Gimenez-Roqueplo and R. R. d. Krijger (2011). "**SDHA Immunohistochemistry Detects Germline SDHA Gene Mutations in Apparently Sporadic Paragangliomas and Pheochromocytomas**." *The Journal of Clinical Endocrinology & Metabolism* **96**: 1472-1476.

Korpershoek, E., K. Pacak and L. Martiniova (2012). "**Murine Models and Cell Lines for the Investigation of Pheochromocytoma: Applications for Future Therapies?**" *Endocrine Pathology* **23**: 43-54.

Kuehnel, W. (2003). **Color Atlas of Cytology, Histology, and Microscopic Anatomy**, Thieme.

Kunst, H. P. M., M. H. Rutten, J.-P. d. Monnink, L. H. Hoefsloot, H. J. L. M. Timmers, H. A. M. Marres, J. C. Jansen, H. Kremer, J.-P. Bayley and C. W. R. J. Cremers (2011). "**SDHAF2 (PGL2-SDH5) and Hereditary Head and Neck Paraganglioma**." *Clinical Cancer Research* **17**: 247-254.

Lee, S. C., S. B. Chionh, S. M. Chong and P. E. Taschner (2003). "**Hereditary paraganglioma due to the SDHD M1I mutation in a second Chinese family: a founder effect?**" *The Laryngoscope* **113**: 1055-1058.

Linos, D. A. and J. A. v. Heerden (2005). **Adrenal Glands: Diagnostic Aspects and Surgical Therapy**, Springer.

Lodish, H., A. Berk and S. L. Zipursky (2000). Electron Transport and Oxidative Phosphorylation. Molecular Cell Biology. W. H. Freeman.

López-Lázaro, M. (2008). "**The Warburg Effect: Why and How do Cancer Cells Activate Glycolysis in the Presence of Oxygen**." *Anti-Cancer Agents in Medicinal Chemistry* **8**: 305-312.

Metzler, D. E. (2004). **Biochemistry - The Chemical Reactions Of Living Cells**, Elsevier.

Mulligan, L. M., C. Eng, C. S. Healey, D. Clayton, J. B. J. Kwok, E. Gardner, M. A. Ponder, A. Frilling, C. E. Jackson, H. Lehnert, H. P. H. Neumann, S. N. Thibodeau and B. A. J. Ponder (1994). "**Specific mutations of the RET proto-oncogene are related to disease phenotype in MEN 2A and FMTC**." *Nature Genetics* **6**: 70 - 74.

Nakamura, E., P. Abreu-e-Lima, Y. Awakura, T. Inoue, T. Kamoto, O. Ogawa, H. Kotani, T. Manabe, G.-J. Zhang, K. Kondo, V. Nosé and W. G. Kaelin (2006). "**Clusterin Is a Secreted Marker for a Hypoxia-Inducible Factor-Independent Function of the von Hippel-Lindau Tumor Suppressor Protein**." *American Journal of Pathology* **168**: 574-584.

Nederveen, F. H. v., J. Gaal, J. Favier, E. Korpershoek, R. A. Oldenburg, E. M. C. A. d. Bruyn, H. F. B. M. Sleddens, P. Derkx, J. Rivière, H. Dannenberg, B.-J. Petri, P. Komminoth, K. Pacak, W. C. J. Hop, P. J. Pollard, M. Mannelli, J.-P. Bayley, A. Perren, S. Niemann, A. A. Verhofstad, A. P. d. Bruïne, E. R. Maher, F. Tissier, T. Méatchi, C. Badoual, J. Bertherat, L. Amar, D. Alataki, E. V. Marck, F. Ferrau, J. François, W. W. d. Herder, M.-P. F. M. V. Peeters, A. v. Linge, J. W. M. Lenders, A.-P. Gimenez-Roqueplo, R. R. d. Krijger and W. N. M. Dinjens (2009). "**An immunohistochemical procedure to detect patients with**

paraganglioma and phaeochromocytoma with germline SDHB, SDHC, or SDHD gene mutations: a retrospective and prospective analysis." *The Lancet Oncology* **10**: 764–771.

Neumann, H. P. H., M. Sullivan, A. Winter, A. Malinoc, M. M. Hoffmann, C. C. Boedeker, H. Bertz, M. K. Walz, L. C. Moeller, K. W. Schmid and C. Eng (2011). "**Germline Mutations of the TMEM127 Gene in Patients with Paraganglioma of Head and Neck and Extraadrenal Abdominal Sites**." *The Journal of Clinical Endocrinology & Metabolism* **96**: 1279–1282.

Nussey, S. and S. Whitehead (2001). **Endocrinology: An Integrated Approach**.

Pigny, P., A. Vincent, C. C. Bauters, M. Bertrand, V. T. d. Montpreville, M. Crepin, N. Porchet and P. Caron (2008). "**Paraganglioma after Maternal Transmission of a Succinate Dehydrogenase Gene Mutation**." *The Journal of Clinical Endocrinology & Metabolism* **93**: 1609-1615.

Qin, Y., L. Yao, E. E. King, K. Buddavarapu, R. E. Lenci, E. S. Chocron, J. D. Lechleiter, M. Sass, N. Aronin, F. Schiavi, F. Boaretto, G. Opocher, R. A. Toledo, S. P. A. Toledo, C. Stiles, R. C. T. Aguiar and P. L. M. Dahia (2010). "**Germline mutations in TMEM127 confer susceptibility to pheochromocytoma**." *Nature Genetics* **42(3)**: 229-233.

Quayle, F. J., E. A. Fialkowski, R. Benveniste and J. F. Moley (2007). "**Pheochromocytoma penetrance varies by RET mutation in MEN 2A**." *Surgery* **142**: 800-805.

Ranieri, G. and G. Gasparini (2001). **Metastasis Research Protocols : Analysis of Cells and Tissues**, Springer.

Rapizzi, E., T. Ercolino, L. Canu, V. Giaché, M. Francalanci, C. Pratesi, A. Valeri and M. Mannelli (2012). "**Mitochondrial function and content in pheochromocytoma/paraganglioma of succinate dehydrogenase mutation carriers**." *Endocrine-Related Cancer* **19**: 261-269.

Raue, F. and K. Frank-Raue (2010). "**Update multiple endocrine neoplasia type 2**." *Familial Cancer* **9**: 449–457.

Ribon, V., T. Leff and A. R. Saltiel (1994). "**c-Myc does not require max for transcriptional activity in PC-12 cells.**" *Molecular and Cellular Neurosciences* **5**: 277-282.

Ricketts, C., E. R. Woodward, P. Killick, M. R. Morris, D. Astuti, F. Latif and E. R. Maher (2008). "**Germline SDHB Mutations and Familial Renal Cell Carcinoma**." *Journal of the National Cancer Institute* **100**: 1260 – 1262

Ricketts, C. J., J. R. Forman, E. Rattenberry, N. Bradshaw, F. Lalloo, L. Izatt, T. R. Cole, R. Armstrong, V. K. Kumar, P. J. Morrison, A. B. Atkinson, F. Douglas, S. G. Ball, J. Cook, U. Srirangalingam, P. Killick, G. Kirby, S. Aylwin, E. R. Woodward, D. G. Evans, S. V. Hodgson, V. Murday, S. L. Chew, J. M. Connell, T. L. Blundell, F. Macdonald and E. R. Maher (2010). "**Tumor risks and genotype-phenotype-proteotype analysis in 358 patients with germline mutations in SDHB and SDHD**." *Human Mutation* **31**: 41-51.

Simi, L., R. Sestini, P. Ferruzzi, M. S. Gaglianò, F. Gensini, M. Mascalchi, L. Guerrini, C. Pratesi, P. Pinzani, G. Nesi, T. Ercolino, M. Genuardi and M. Mannelli (2005). "**Phenotype variability of neural crest derived tumours in six Italian families segregating the same founder SDHD mutation Q109X**." *Journal of Medical Genetics* **42**: 1-7.

Smeitink, J. A. M. (2004). **Oxidative Phosphorylation in Health and Disease**, Springer.

Smith, E. H., R. Janknecht and L. J. Maher (2007). "**Succinate inhibition of a-ketoglutarate-dependent enzymes in a yeast model of paraganglioma**." *Human Molecular Genetics* **16**: 3136–3148.

Squire, L., D. Berg, F. E. Bloom, S. d. Lac, A. Ghosh and N. C. Spitzer (2008). **Fundamental Neuroscience**, Elsevier.

Taschner, P. E., J. C. Jansen, B. E. Baysal, A. Bosch, E. H. Rosenberg, A. H. Bröcker-Vriends, A. G. v. D. Mey, G. J. v. Ommen, C. J. Cornelisse and P. Devilee (2001). "**Nearly all hereditary paragangliomas in the Netherlands are caused by two founder mutations in the SDHD gene.**" *Genes Chromosomes and Cancer* **31**: 274-281.

Thompson, L. D. R. (2002). "**Pheochromocytoma of the Adrenal Gland Scaled Score (PASS) to Separate Benign From Malignant Neoplasms - A Clinicopathologic and Immunophenotypic Study of 100 Cases**." *Lippincott Williams & Wilkins* **26**: 551-566.

Vecchi, A. (1994). "**Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium.**" *European Journal of Cell Biology* **63**: 247-254.

Velasco, A., V. Palomar-Asenjo, L. Gañan, L. Catasus, N. Llecha, A. Panizo, V. Palomar-Garcia, M. Quer and X. Matias-Guiu (2005). "**Mutation analysis of the SDHD gene in four kindreds with familial paraganglioma: description of one novel germline mutation.**" *Diagnostic Molecular Pathology* **14**: 109-114.

Weidner, N. (1995). "**Intratumor Microvessel Density as a Prognostic Factor in Cancer**." *American Journal of Pathology* **147**: 9-19.

Welander, J., C. Larsson, M. Bäckdahl, N. Hareni, T. Sivlér, M. Brauckhoff, P. Söderkvist and O. Gimm (2012). "**Integrative genomics reveals frequent somatic NF1 mutations in sporadic pheochromocytomas**." *Human Molecular Genetics*: 1-11.

Welander, J., P. Söderkvist and O. Gimm (2011). "**Genetics and clinical characteristics of hereditary pheochromocytomas and paragangliomas**." *Endocrine-Related Cancer* **18**: 253 - 276.

Yao, L., F. Schiavi, A. Cascon, Y. Qin, L. Inglada-Pérez, E. E. King, R. A. Toledo, T. Ercolino, E. Rapizzi, C. J. Ricketts, L. Mori, M. Giacchè, A. Mendola, E. Taschin, F. Boaretto, P. Loli, M. Iacobone, G.-P. Rossi, B. Biondi, J. V. Lima-Junior, C. E. Kater, M. Bex, M. Vikkula, A. B. Grossman, S. B. Gruber, M. Barontini, A. Persu, M. Castellano, S. P. A. Toledo, E. R. Maher, M. Mannelli, G. Opocher, M. Robledo and P. L. M. Dahia (2012). "**Spectrum and Prevalence of FP/TMEM127 Gene Mutations in Pheochromocytomas and Paragangliomas**." *American Medical Association* **304**(23): 2611-2619.

Young, W. F. and N. M. Kaplan (2012) **Pheochromocytoma in genetic disorders**.

Zhu, J., J. Blenis and J. Yuan (2008). "**Activation of PI3K/Akt and MAPK pathways regulates Myc-mediated transcription by phosphorylating and promoting the degradation of Mad1**." *Proceedings of the National Academy of Sciences* **105**: 6584 – 6589.

Zielke, A., M. Middeke, S. Hoffmann, M. Colombo-Benkmann, P. Barth, I. Hassan, A. Wunderlich, L. C. Hofbauer and Q.-Y. Duh (2002). "**VEGF-mediated angiogenesis of human pheochromocytomas is associated to malignancy and inhibited by anti-VEGF antibodies in experimental tumors**." *Surgery* **132**: 1056-1063.